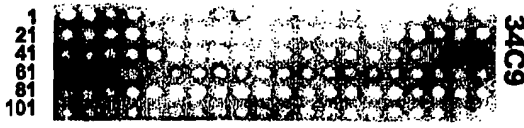

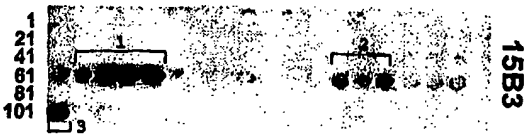




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : C12N 15/70, C07K 16/18, C12N 5/20, C07K 14/47, C12N 15/06, 1/21, 5/10, G01N 33/577, 33/68, A61K 39/395</p>	A1	<p>(11) International Publication Number: WO 98/37210</p> <p>(43) International Publication Date: 27 August 1998 (27.08.98)</p>
<p>(21) International Application Number: PCT/EP98/00917</p> <p>(22) International Filing Date: 18 February 1998 (18.02.98)</p> <p>(30) Priority Data: 97102837.8 21 February 1997 (21.02.97) EP</p> <p>(34) Countries for which the regional or international application was filed: AT et al.</p> <p>(71) Applicant (for all designated States except US): KANTON ZÜRICH vertreten durch DIE ERZIEHUNGSDIREKTION [CH/CH]; Walchetur, CH-8090 Zürich (CH).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): KORTH, Carsten [DE/CH]; Lengstrasse 70, CH-8008 Zürich (CH). STIERLI, Beat [CH/CH]; Alte Landstrasse 38, CH-8114 Dänikon (CH). STREIT, Peter [CH/CH]; Scheuchzerstrasse 9, CH-8006 Zürich (CH). OESCH, Bruno [CH/CH]; Haldenstrasse 13, CH-5233 Stilli (CH). MOSER, Markus [CH/CH]; Waidfussstrasse 25, CH-8037 Zürich (CH).</p> <p>(74) Agent: SCHAEFER, Konrad; Schaefer & Emmel, Gehölzweg 20, D-22043 Hamburg (DE).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BG, BR, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KP, KR, LK, LR, LS, LT, LU, LV, MK, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TR, UA, UG, US, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
<p>(54) Title: IMMUNOLOGICAL DETECTION OF PRIONS</p> <p>(57) Abstract</p> <p>The presented invention relates to monoclonal antibodies useful in sensitive and specific immunological assays for the identification of prions in various tissues and body fluids, the production of such monoclonal antibodies by means of immunisation of PrP^{0/0} mice by means of a new recombinant fragment of PrP and the use of the antibodies, e.g. for therapeutic and preventive treatments of humans and animals suffering from prion diseases.</p> <div style="display: flex; justify-content: space-around; align-items: flex-start;"> <div style="text-align: center;"> <p>A</p>  <p>Peptides 59-63 comprising amino acids 149-153 of BoPrP</p> </div> <div style="text-align: center;"> <p>B</p>  <p>Peptides 64-86 comprising amino acids 155-163 of BoPrP</p> </div> <div style="text-align: center;"> <p>C</p>  <p>Peptides 62-85 comprising amino acids 163-169 of BoPrP Peptides 73-76 comprising amino acids 173-181 of BoPrP Peptide 102 comprising amino acids 226-237 of BoPrP</p> </div> </div>		

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Immunological Detection of Prions

5

Field of the Invention

The present invention relates to monoclonal antibodies reacting with certain epitopes of recombinant bovine prion protein, native and denatured normal or disease-specific prion proteins in soluble or insoluble state, stable hybridoma cell lines producing these monoclonal antibodies, recombinant expression vectors for the expression of recombinant bovine prion protein, purified recombinant bovine prion protein, a test kit for the diagnosis of prion diseases, diagnostic methods for the immunological detection of prion diseases, pharmaceutical preparations for the prevention and therapy of prion diseases, a method for clearing biological material from infectious prion proteins, and methods for the production of these materials.

Abbreviations used hereinbefore and hereinafter are the following:

BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CSF	cerebrospinal fluid
CJD	Creutzfeldt-Jakob disease,
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELIFA	enzyme linked immuno filtration assay
ELISA	enzyme linked immuno sorbent assay
Fab	fragment of antibody digested with papain
(Fab') ₂	fragment of antibody digested with pepsin
FFI	Fatal Familial Insomnia
GPI-anchor	glycolipid-anchor which „ties“ PrP to the outside of the cell membrane
GSS	Gerstmann-Sträussler-Scheinker disease
H(A)T-medium	hypoxanthine-(aminopterin)-thymidine medium

HEPES	hydroxyethyl-piperazineethane sulfonic acid
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
IPTG	isopropyl- β -D-thiogalactoside
mAB	monoclonal antibody
MOPS	morpholinepropanesulfonic acid
NC	nitrocellulose membrane
o/n	overnight
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
prion	proteinaceous infectious particle; the infectious agent of prion diseases, supposedly consisting at least of PrP ^{Sc} and maybe another yet unknown molecule
PrP	prion protein; refers to the common amino acid sequence rather than to a distinct conformation of the two prion protein isoforms
PrP ^{0/0} -mice	mice lacking a functional PrP gene
PrP ^C	a normal host prion protein of unknown function; apparent molecular weight 33-35 kDa, same amino acid chain, and same glycosylation at two asparagine residues as PrP ^{Sc} , is after proteinase K treatment fully digested.
PrP ^{Sc}	the disease-specific, abnormal isoform of PrP ^C , with the same amino acid chain, apparent molecular weight 33-35 kDa, glycosylated at two asparagine residues, is after proteinase K treatment shortened to a 27-30 kDa C-terminal fragment. Species-specific PrP ^{Sc} isoforms term: human PrP ^{Sc} (instead of PrP ^{CJD}), bovine PrP ^{Sc} (instead of PrP ^{BSE}) etc.
rbPrP	recombinant bovine prion protein (amino acids 25 to 242 of the bovine PrP gene according to Goldmann et al. 1991; with an additional N-terminal methionine) expressed in E. coli comprising the bovine PrP open reading frame except for the N-terminal signal sequence and the C-terminal GPI-anchor sequence; both are cleaved of during cellular processing. Since this protein is not glycosylated it has a molecular weight

of 23 kD.

RT	room temperature
SAF	scrapie-associated fibrils; same as rods: plaque-like multimeric PrP ^{Sc} aggregates
SDS	sodium dodecyl sulfate
TBST	Tris-buffered saline, Tween 20
TMB	tetramethylbenzidine

Prion diseases are transmissible neurodegenerative diseases of the central nervous system (for review see Prusiner, 1991). They can be transmitted, inherited or occur
 5 sporadically and are observed in animals (e.g. bovine spongiform encephalopathy [BSE] in cattle, scrapie in sheep) as well as in humans (Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, Fatal Familial Insomnia, Kuru). Prion diseases have a characteristically long incubation period and, with the onset of clinical symptoms, lead to ataxia, dementia, psychiatric disturbances and sleeplessness before inevitable death occurs.
 10 Neuropathological changes include vacuolar degeneration of brain tissue, astrogliosis and amyloid plaque formation. In the infected subjects, neither a systemic immune response, nor an obvious specific immune response like antibody production to PrP has been observed (Kasper et al., 1982; Garfin et al., 1978) however, some unspecific activation of immune cells in the brain was reported (Williams et al., 1995; Williams et al., 1994).

15 The infectious agent appears to exist in a variety of strains, which cause distinct incubation times and histopathology (Bruce et al., 1994; Hecker et al., 1992). Transmission of prion diseases is possible between species and most easily within the same species (Prusiner, 1991).

The infectious agent, the prion, is associated with a disease-specific protein, PrP^{Sc},
 20 that is an abnormal isoform of a host protein, PrP^C (Oesch et al., 1985; Basler et al., 1986). Both, PrP^{Sc} and PrP^C, have an apparent molecular weight of 33-35 kDa on SDS-polyacrylamide gels. They have the same amino acid sequence and are glycosylated at two asparagine residues (Oesch et al., 1985). After proteinase K treatment, PrP^{Sc} is shortened

to a characteristic 27-30 kDa fragment while PrP^C is fully digested (Bolton et al., 1982; Oesch et al., 1985); this led to the conclusion that the disease-specific isoform PrP^{Sc} is partially protease resistant while the normal host isoform PrP^C is not.

Studies on the synthesis and localization of the two PrP isoforms in cultured
5 cells have shown that PrP^C is attached to the cell surface by a glycosyl phosphatidylinositol (GPI) anchor while PrP^{Sc} accumulates intracellularly within cytoplasmic vesicles (Stahl et al., 1987). Another difference between PrP^C and PrP^{Sc} is reflected in their three-dimensional structure: PrP^{Sc} has less alpha helical secondary structures and increased beta sheet content as compared to PrP^C (Pan et al., 1993). So far, no chemical differences
10 between the two isoforms have been observed (Stahl et al., 1993). In summary, PrP^{Sc} and PrP^C have the same amino acid sequence but a different folding. The misfolded prion protein is associated with infectivity and neurotoxicity.

The infectious agent is inactivated by treatments which denature proteins while reagents destroying nucleic acids have no effect (Diener et al., 1982; Alper et al., 1978). In
15 addition, no single nucleic acid capable for coding a protein has been purified until date (Riesner et al., 1993). This has lead to the hypothesis that PrP^{Sc} itself might comprise the infectious particle (Griffith, 1967; Prusiner, 1982). According to this hypothesis, replication of infectivity is achieved by the replication of the pathogenic conformation. It is supposed that infectious PrP^{Sc} molecules convert the normal host protein PrP^C to the PrP^{Sc}
20 conformation (Cohen et al., 1994). Conversion of PrP^C to PrP^{Sc} was claimed to have been achieved *in vitro* thereby mimicking species and strain characteristics comparable to the conversion dynamics *in vivo* (Kocisko et al., 1994; Bessen et al., 1995). However, these *in vitro* converted PrP^{Sc} molecules have, to date, not shown to be infectious.

The function of the normal host protein, PrP^C, is unknown. Mice devoid of PrP^C
25 are viable and show no obvious signs of neurological and physical impairment (Bueler et al., 1992). In addition, these mice are not susceptible to infection with prions, underlining the central importance of PrP in the replication of infectivity and/or pathology of these diseases (Bueler et al., 1993; Prusiner et al., 1993). More subtle investigations of PrP knockout mice revealed impaired synaptic function (Collinge et al., 1994) and altered sleep regulation

(Tobler et al., 1996). However, a molecular function of PrP^C could not be deduced from these findings.

Prion diseases have gained public interest with the appearance of BSE in the early
5 eighties in Great Britain (Hope et al., 1988); for review see (Wells and Wilesmith, 1995).
The disease is supposed to have been transmitted by feeding prion-contaminated meat and
bone meal to cattle. It is thought that BSE prions originated from scrapie-diseased sheep by
crossing the species barrier from sheep to cattle. BSE has caused an epidemic of
considerable importance for both, public health and cattle-dependent economy. Remarkably,
10 no diagnostic method suitable for mass screening of infected tissues of cattle has been
developed to date.

Initial diagnosis of prion diseases classically relies on the appearance of clinical
symptoms. A definitive diagnosis is made by the observation of neuropathological changes
in the medulla oblongata. In few cases, BSE has been shown to be transmissible to other
15 cattle, sheep, pigs and mice. Modern diagnosis additionally uses immunological detection of
PrP^{Sc} in brain sections. Since PrP^{Sc} can be detected in the CNS after half of the incubation
time in experimentally infected laboratory animals (Jendroska et al., 1991; Hecker et al.,
1992), it may serve as an early marker of infection. Hence, specific and sensitive detection
of PrP^{Sc} allows the identification of infected animals at a subclinical stage and will help to
20 reduce possible human health risks. By autumn 1996, the BSE epidemic has killed over
160'000 cows in Great Britain alone. In the absence of a diagnostic test, only cattle with
clinical symptoms were sorted out from being further processed, allowing a great number of
BSE- infected cattle to enter the human food chain (Anderson et al., 1996). This lead to the
suspicion that the appearance of a new variant of Creutzfeldt-Jakob disease in Great Britain
25 was caused by transmission of BSE to humans (Will et al., 1996; Collinge et al., 1996). A
sensitive detection method for bovine PrP^{Sc} will allow the identification and removal of
subclinical BSE-cases from the human food chain.

Oesch et al. (1994) have used a procedure that allows to quantitate the disease-
specific isoform of PrP in hamsters. The procedure is based on an ELIFA (enzyme-linked
30 immuno-filtration assay), and is adapted to the particularities of the prion protein, especially
the poor solubility of the disease-specific isoform that has made application of conventional
ELISA techniques difficult. This procedure (described in detail below) allows for testing of

thousands of samples and is thus appropriate for routine screening of animals and humans for prion diseases.

Tagliavini et al. (WO 93/23432) describe a method for detecting soluble prion polypeptides. The drawback of this method is that the inventors claim to detect prion polypeptides that are
5 soluble in vivo, however, it is known since a long time that the disease-associated prion protein PrP^{Sc} is insoluble in vivo. State of the art is that insoluble PrP^{Sc} has to be solubilized in vitro to be detected by immunological methods. Tagliavini et al state (page 3, row 31) „...such truncated scrapie proteins have not been found to exist in vivo in substantially soluble form“. Furthermore, the inventors give an example wherein they show
10 soluble prion polypeptide fragments in the cerebrospinal fluid (CSF) of patients that do not suffer of the human prion disease CJD but of other unrelated diseases. However, the inventors do not show in vivo soluble protease-resistant prion polypeptides which would prove their hypothesis about the existence of disease-specific prion polypeptides in CSF. In addition, to show prion polypeptides in CSF they use an immunoblot (Western blot); this
15 technique is not appropriate to detect naturally occurring soluble prion polypeptides, since the immunoblot technique requires solubilization of proteins in vitro prior to gel electrophoresis. This procedure would then solubilize even insoluble prion polypeptides that would be suspended in CSF.

Major shortcomings for the immunological detection of PrP have been the
20 unavailability of excellent antibodies able to detect the **native** disease-specific prion protein (Kascsak et al., 1987; Barry and Prusiner, 1986; Takahashi et al., 1986; Barry et al., 1986). In particular, native PrP^{Sc} was invisible to antibodies (Serban et al., 1990). Furthermore, no monoclonal antibodies recognizing the bovine PrP were available. The reason for the difficulties in raising monoclonal as well as polyclonal antibodies is the highly conserved
25 amino acid sequence of PrP in mammals which apparently prevents an antibody response against most epitopes.

Kascsak et al., (1987) describe the monoclonal antibody 265K3F4 produced by hybridoma cell line ATCC HB 9222 directed against scrapie-associated fibril proteins. The drawback of this method is that by immunizing wild-type mice with PrP, due to self-tolerance, an
30 antigenic reaction against many epitopes is suppressed. The inventors immunized wild-type mice with purified scrapie-associated fibrils (SAF); SAF are multimeric complexes consisting of PrP^{Sc} that are purified by a ultracentrifugation. The inventors describe an

antibody, termed 3F4, that binds only to hamster and human PrP. Furtheron, the antigen has to be denatured either by formic acid or SDS to be detected. It is stated (Kascsak et al., 1987) that the 3F4 antibody binds to undenatured SAF 10-fold weaker than to formic acid-denatured SAF. However, the 3F4 antibody does not distinguish between PrP^C and PrP^{Sc}.

5 Williamson et al. (1996) have tried to circumvent the lack of an immune response to a highly conserved protein by immunizing transgenic mice lacking PrP (PrP^{0/0}-mice) with PrP, however, without success. These authors state that after immunizing PrP^{0/0}-mice with PrP „... killing these mice for hybridoma production has repeatedly yielded hybridoma cells that failed to secrete anti-PrP antibodies beyond a period of 48h“. They presume that during
10 the 48 hours after the fusion anti-PrP antibody-secreting clones either are suppressed to secrete further antibodies or die because of an interaction of the secreted antibodies with cell-resident PrP. Williamson et al tried to circumvent this problem by isolating antibody-coding RNA and constructing recombinant antibodies by the phage display technique. They obtained several recombinant antibodies which bind to non-denatured mouse prion rods
15 (PrP^{Sc}) in the ELISA technique, however, much weaker than to denatured rods and only if substantial amounts of rods were bound to the wells (0.2µg/well incubated with 5µg/ml antibody). However, these recombinant antibodies do not detect native PrP^{Sc} in non-denatured histoblots. Thus, the necessity of purifying PrP^{Sc} before antibody detection complicates the use of their immunological detection method.

20 Krasemann et al. (1996) have made monoclonal antibodies by means of immunizing PrP^{0/0}-mice. After DNA-immunization by injecting the DNA coding for the human prion protein directly into a regenerating muscle the mice were subsequently boosted with Semliki Forest Virus particles containing recombinant human prion protein. The authors present hybridoma cell lines producing monoclonal antibodies that bind to the native
25 and denatured normal human prion protein. The binding of these antibodies to the native or denatured disease-specific prion protein, however, is not demonstrated. Furtheron, the obtained antibodies bind to a peptide ELISA system, however an ELISA to normal or disease-specific prion protein is not shown.

We are now the first to show that immunization of PrP knockout mice with
30 highly purified recombinant PrP followed by fusion of splenocytes from these mice with myeloma cells resulted in hybridoma cell lines that secrete highly specific antibodies to both

PrP isoforms (PrP^C and PrP^{Sc}) in their native as well as denatured state. On the basis of these antibodies, highly specific immunological testing for prion disease was developed.

Object of the Invention

5 It is an object of the present invention to overcome the drawbacks and failures of prior art and to provide monoclonal antibodies from stable hybridoma cell lines which can be used in the diagnosis and therapy of prion diseases.

Summary of the invention

10 Surprisingly the drawbacks of the prior art can be overcome by immunization of PrP^{0/0} knockout mice with highly purified recombinant PrP followed by fusion of splenocytes from these mice with myeloma cells. The resulting hybridoma cell lines are surprisingly stable and secrete highly specific antibodies to both PrP isoforms (PrP^C and PrP^{Sc}) in their native as well as denatured state. The obtained antibodies are very useful for
15 the development of highly specific immunological tests for prion diseases and other purposes.

The present invention concerns a monoclonal antibody or a fragment thereof capable of specifically binding to recombinant bovine prion protein, and native and denatured normal PrP^C or disease-specific prion protein PrP^{Sc} in an antigen-antibody complex.

20 The present invention concerns further an antibody or a fragment thereof capable of specifically binding to the binding region (idiotype) of said antibody.

The present invention concerns further a hybridoma cell line capable of producing a monoclonal antibody capable of specifically binding to recombinant bovine prion protein, and native and denatured normal PrP^C or disease-specific prion protein PrP^{Sc} in an
25 antigen-antibody complex.

The present invention concerns further a recombinant expression vector for the expression of recombinant bovine prion protein.

The present invention concerns further a highly purified recombinant bovine prion protein, which may be in reduced or oxidized form.

30 The present invention concerns further a method for the production of an antibody as mentioned above, comprising culturing a hybridoma cell line as mentioned above and isolating the monoclonal antibody from the supernatant.

The present invention concerns further a method for the production of a hybridoma cell line as mentioned above, comprising administering to PrP^{0/0} mice (knockout mice without a functional PrP gene) an immunizing amount of recombinant prion protein as mentioned above, removing the spleen from the immunized mice, recovering splenocytes
5 therefrom, fusing the latter with P3X63Ag8U.1 hybridoma cells ATCC CRL 1597, growing the cells in a selection medium, screening the cells with recombinant PrP and isolating the positive cells.

The present invention concerns further a method for the production of an expression vector as mentioned above, comprising amplifying DNA from bovine genomic DNA coding
10 for PrP by means of N- and C-terminal primers, and inserting the amplified DNA coding for PrP in the correct reading frame into an expression vector.

The present invention concerns further a method for the production of recombinant bovine prion protein comprising culturing microorganisms or cell lines with an expression vector as mentioned above in an appropriate culture medium and isolating and purifying the
15 recombinant protein.

The present invention concerns further a test kit for the diagnosis of prion diseases.

The present invention concerns further an immunological detection procedure for the diagnosis of disease-specific prion proteins.

The present invention concerns further a pharmaceutical preparation for the therapy
20 and prevention of prion diseases comprising a monoclonal antibody as mentioned above and pharmaceutical carrier.

The present invention concerns further a method for the therapy or prevention of prion diseases comprising administering to a patient suffering from such disease or being likely to becoming a victim of this disease a therapeutical or preventive amount of a
25 monoclonal antibody as mentioned above.

The present invention concerns further a method for clearing biological material from prions comprising treating said material with a monoclonal antibody as mentioned above.

Brief Description of the Drawings

Figure 1. a. Western blot of bovine PrP^C/PrP^{Sc} without (0µg) and after treatment with 20, 40, 80 or 100µg/ml of proteinase K for 1h at 37°C. The blot shows that bovine PrP^{Sc} in homogenates from thalamus (left) and spinal cord (right) of BSE-diseased and normal cattle is protease-resistant at several concentrations as compared to bovine PrP^C. Staining with mAB 6H4 followed by peroxidase-labelled anti-mouse IgG antibody. Peroxidase activity was detected by chemiluminescence.

b. Western blot of different tissue homogenates from normal cattle. PrP in white blood cells is recognized by the mAB 34C9.

Figure 2. Western blots of brain homogenates from different species.

a: mAB 6H4 stains PrP of all depicted species,
b: mAB 34C9 does not stain PrP from hamster and sheep; mouse PrP staining is weak. This differential staining is consistent with the sequence homology of the mapped epitopes of PrP from different species.

Figure 3. a. ELIFA standard curve. The standard curve shows a linear relation between the concentration of recombinant bovine PrP and the OD₄₅₀ with a background below 0.5 ng/ml rbPrP. Detection was with mAB6H4.

b. Results ELIFA. Both, the brain homogenates from normal and BSE-diseased cattle have high total amount of PrP as measured by the OD₄₅₀. However, while in BSE-brain there is a substantial amount of protease K-resistant PrP^{Sc}, no such PrP can be detected in normal brain.

c. Results ELISA. Both, the brain homogenates from normal and BSE-diseased cattle have high total amount of PrP as measured by the OD₄₅₀. However, while in BSE-brain there is a substantial amount of protease K-resistant PrP^{Sc}, no such PrP can be detected in normal brain.

Empty column: without proteinase K treatment

black column: after proteinase K treatment

Figure 4. Scheme capture ELISA and multimeric PrP^{Sc} See also text.

a.: monomeric PrP^C has no additional binding sites since the only binding site is occupied by the coating antibody.

b.: multimeric disease-specific PrP^{Sc} has additional binding sites for the detecting,
5 peroxidase-labeled mAB 6H4 (POD) or 15B3 (POD).

Figure 5. Schematic map of plasmid pbPrP3. The insert PrP ORF corresponds to SEQ ID NO. 1 with the restriction sites Nde I and BamH I.

10 **Figure 6. a.** Western blots of normal bovine brain homogenates and recombinant bovine PrP. mABs 6H4 and 34C9 recognize both bovine PrP and rbPrP, whereas mAB 15B3 recognizes only rbPrP. Recombinant rbPrP runs lower and sharper than PrP from brain homogenates because it is not glycosylated.

b. Conformation-sensitive ELIFA of brain homogenates from BSE-diseased and
15 normal cattle at different denaturation states. Staining with monoclonal antibody 15B3. In the native state, mAB 15B3 stains only bovine PrP^{Sc}, and not PrP^C.

Figure 7. Epitope mapping on the peptide library for bovine PrP:

a.: mAB 34C9 recognizes peptides Nos. 59 to 63 of the peptide library comprising
20 the epitopes of amino acids 149 to 153 (Leu Ile His Phe Gly; SEQ ID NO 5) of bovine PrP according to Goldmann et al. (1991) corresponding to amino acids 126-130 in SEQ ID NO 2.

b.: mAB 6H4 recognizes peptides Nos. 64 to 66 of the peptide library comprising
the epitopes of amino acids 155 to 163 (Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu; SEQ ID
25 NO 6) of bovine PrP according to Goldmann et al. (1991) corresponding to amino acids 132-140 in SEQ ID NO. 2.

c.: mAB 15B3 recognizes 3 distinct arrays of peptides: Nos. 62 to 65 of the peptide
library comprising amino acids 153 to 159 (Gly Ser Asp Tyr Glu Asp Arg; SEQ ID NO 7),
Nos. 73 to 75 comprising amino acids 173 to 181 (Tyr Tyr Arg Pro Val Asp Gln Tyr Ser;
30 SEQ ID NO 8) and No. 102 comprising amino acids 225 to 237 (Cys Ile Thr Gln Tyr Gln
Arg Glu Ser Gln Ala Tyr Tyr; SEQ ID NO 9) of bovine PrP according to Goldmann et al.
(1991).

Figure 8. Immunoprecipitation of bovine, mouse and human PrP with monoclonal antibodies 15B3 and 6H4: **a.** The supernatant of a centrifuged homogenate from the medulla of two different BSE-diagnosed or two normal animals was incubated with antibodies 6H4 or 15B3. Antibodies were precipitated with protein A (15B3) or protein G-agarose (6H4). As a control, protein A only was incubated without antibodies. Precipitates were analysed on a western blot for the presence of PrP using a polyclonal rabbit antiserum to bovine PrP and goat-anti-rabbit Ig coupled to alkaline phosphatase. Signals were developed with chemiluminescence substrates. Crossreaction of the secondary antibody with immunoprecipitated mouse immunoglobulins leads to the prominent band at about 50K. Note the 50 K band characteristic for PrP^{Sc} in the 15B3 but not in the 6H4 immunoprecipitations. **b.** Proteinase K digestion of PrP^{BSE} immunoprecipitated with mAb 15B3. Undigested and digested bovine brain homogenates were compared to proteinase K digested immunoprecipitates with protein A-agarose only or with 15B3. The sharp band at 31K represents a crossreactivity of the secondary antibody with proteinase K. The same immunoprecipitates and method of analysis were used as in a. **c.** Immunoprecipitation of mouse PrP^{Sc} with mAb 15B3. Homogenates from PrP-null mice (0/0) or wild-type mice (normal (*/*) or scrapie infected (+/+Sc)) were immunoprecipitated with mAb 15B3 or protein A-agarose only and analysed by western blotting as described. Digestion with proteinase K after (a) or before (b) the immunoprecipitation is indicated. Detection of PrP was done as described. **d.** Immunoprecipitation of human PrP^{CJD} with mAb 15B3. Brain homogenates (cerebellum) from normal persons or CJD patients type 1 (ref. 10) were immunoprecipitated and analysed as described for a. Two representative examples from a total of 4 normal persons and 4 CJD cases are shown.

25

SEQ ID NO 1 shows the 660 base pair sequence encoding the bovine rPrP obtained from genomic bovine DNA by PCR amplification.

SEQ ID NO 2 shows the amino acid sequence of recombinant bovine rPrP which comprises amino acids 25 to 242 of the bovine PrP open reading frame (Goldmann et al., 1991).

30 SEQ ID NO 3 shows the N-terminal sense primer, and

SEQ ID NO 4 shows the C-terminal antisense primer. These primers comprise a Nde I restriction site at the 5'-end and a BamH I restriction site at the 3'-end in the PCR-amplified bovine PrP-DNA.

5 SEQ ID NO 5 shows the amino acid sequence common to peptides 59 to 63 of the peptide library recognized by mAB 34C9.

SEQ ID NO 6 shows the amino acid sequence common to peptides 64 to 66 of the peptide library recognized by mAB 6H4.

SEQ ID NO 7 shows the amino acid sequence common to peptides 62 to 65 of the peptide library being the first of 3 partial sequences recognized by mAB 15 B3.

10 SEQ ID NO 8 shows the amino acid sequence common to peptides 73 to 75 of the peptide library being the second of 3 partial sequences recognized by mAB 15 B3.

SEQ ID NO 9 shows the amino acid sequence common to peptide 102 of the peptide library being the third of 3 partial sequences recognized by mAB 15 B3.

15 Detailed Description of the Invention

In the following detailed description the spirit and scope of the invention will become more clearly explained and understood.

The monoclonal antibodies

20 A monoclonal antibody according to the invention is intended to bind to, to detect and qualitatively and quantitatively measure the presence of epitopes of prion proteins whether they are in soluble or insoluble form in various tissue specimens such as homogenates or sections of brain, spleen, tonsils, white blood cells or others and body fluids such as blood, cerebrospinal fluid saliva, urine or others. The present mABs bind to
25 epitopes of amino acids in a row or to epitopes of amino acids on different loops of the three-dimensional struture of native PrPs which are spatially close to each other. A particular group of the present antibodies binds only to native disease-specific PrP and not to native normal PrP.

Any known mABs which would fall under these definitions are exempted and
30 disclaimed.

The term monoclonal antibody comprises also chimeric monoclonal antibodies having similar properties, which are derived from different animals, such as human/mouse chimeric antibodies or any other chimeric molecule comprising the antigen-binding part of

the monoclonal antibody (idiotype) with other molecules such as antibody fragments of other monoclonal antibodies or enzymes.

A fragment of a monoclonal antibody comprising the binding part of the monoclonal antibody (idiotype) likewise capable of specifically binding the antigen and is termed Fab or (Fab')₂ depending on whether the monoclonal antibody is digested with papain or pepsin, respectively.

A synthetic antibody or fragments thereof designed according to the amino acids or substituted homologous amino acids composing the idiotype responsible for binding the antigen. Homologous amino acids are defined as exchanges within the following five groups: 1. Small aliphatic, nonpolar or slightly polar residues: alanine, serine, threonine, glycine, proline; 2. Polar, negatively charged residues and their amides: aspartic acid, asparagine, glutamic acid, glutamine; 3. Polar, positively charged residues: histidine, arginine, lysine; 4. Large aliphatic, nonpolar residues: methionine, leucine, isoleucine, valine, cysteine; 5. Large aromatic residues: phenylalanine, tyrosine, tryptophan.

Preferred monoclonal antibodies are those named 6H4, 34C9, 15B3 which are produced by hybridoma cell lines DSM ACC2295, DSM ACC2296 and DSM ACC2298, respectively.

The antibodies and fragments thereof are essential tools for immunological detection procedures based on the binding of the prion protein to the presented monoclonal antibodies in an antigen-antibody complex. The monoclonal antibodies of the invention react with recombinant bovine PrP as well as native or denatured PrP^C and PrP^{Sc} whether they are in soluble or insoluble state. The monoclonal antibodies react furtheron with PrP from different species, for example humans, hamsters, pigs, sheep, cattle and mice.

Furthermore, the present antibodies by forming an antigen-antibody complex between the presented monoclonal antibodies and the prion protein can be used to inhibit neurotoxic and infectious properties of the disease-specific prion protein.

Anti-idiotypic antibodies

The invention concerns further anti-idiotypic antibodies which are antibodies that bind with their binding region (idiotype) to the binding region of the original monoclonal antibody. The anti-idiotypic antibody mimicks features of the original antigen, in this case features of PrP. Anti-idiotypic antibodies are raised as polyclonal antibodies (serum) or

monoclonal antibodies from animals immunized with the preferred antibodies according to the invention. Anti-idiotypic antibodies are valuable tools in detecting and blocking interactions of the original antigen (PrP), particularly interactions with receptors and can therefore be used in prevention and therapy of prion diseases.

5

The hybridoma cell lines

A stable hybridoma cell line according to the invention is capable of producing a monoclonal antibody as defined above over a prolonged time period of at least 6 months. Such cell lines are derived from the fusion of a spleen cell expressing the antibody derived
10 from mice lacking a functional PrP gene, and a myeloma cell of mice providing survival of the fused cell line.

Preferred hybridoma cell lines are DSM ACC2295, DSM ACC2296 and DSM ACC2298. The first two cell lines were deposited under the Budapest Treaty on February 06, 1997 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,
15 Mascheroder Weg 1b, D-38124 Braunschweig, a recognized public depository for strains of microorganisms. The hybridoma cell line producing mAB 15B3 was deposited February 13, 1997 unter number DSM ACC2298 at the same depository.

The expression vector for recombinant bovine prion protein

An expression vector for the expression of the recombinant bovine prion protein is a
20 DNA vector, based on the pET11a vector by Novagen comprising essential sequences for expression in the respective host, e.g. a T7-promotor and the DNA coding for the bovine prion protein from codons 25 to 242 with an additional codon ATG at the 5'-end the PrP-coding DNA and sequence for selecting, eg. the ampicillin gene, multiplication and
25 termination..

Preferred expression vector is pbPrP3 as shown in Figure 5.

The recombinant bovine prion protein

The present recombinant bovine prion protein consists of the amino acid sequence
30 ID No:2. It may be unglycosylated or glycosylated.

The present recombinant bovine prion protein PrP is purified to a homogeneity of >98%. It can be present in oxidized or reduced form. In the oxidized form the single -S-S-bridge is present whereas in the reduced form two SH groups are present instead. The

amino acid sequence of the present recombinant bovine PrP is shown by ID No:2. The protein is glycosylated if expressed in a glycosylating eukaryotic cell line, such as Chinese Hamster cells or unglycosylated if expressed in a procaryotic cell line, such as Escherichia coli. Mixtures of oxidized and reduced form are also comprised. The oxidized form has the
5 molecular weight of 23676.8 Da and the reduced form 236886.1 Da as determined by electrospray mass spectroscopy. The present full length recombinant bovine prion protein is unique in terms of its homogeneity, since other groups in the art have reported of being unable to purify homogenous full length recombinant prion protein of other species (Mehlhorn et al., 1996; Riek et al., 1996).

10 The reduced form of the recombinant PrP is particularly interesting since it has been reported to contain more β -sheet secondary structures than the oxidized form (Mehlhorn et al., 1996), hence mimicking structural features of PrP^{Sc}. However, the reduced recombinant isoforms have been reported to be neither protease-resistant nor infectious (Mehlhorn et al., 1996).

15 A native prion protein PrP is the prion protein in a fully folded state, i.e. the three-dimensional structure is present. Only in the native, i.e. folded state PrP isoforms are different (normal native vs. disease-specific native PrP)

A denatured prion protein is the prion protein in the unfolded state. Unfolding is usually achieved by the addition of chaotropic substances such as urea or guanidinium
20 hydrochloride. In the denatured state, both PrP isoforms are irreversibly the same, even if they have been normal native or disease-specific native before.

An antigen-antibody complex is a physical attachment of an antibody or fragment thereof with the corresponding antigen by intermolecular forces because the surfaces match in a unique way. The matching surface on the antibody is called idiotype and the surface on
25 the antigen is called epitope.

Suitable epitopes detectable by the present antibodies are for example linear amino acid sequences having from about 3 to about 15 amino acids in a row or are completely three-dimensional ("patch") in that distant amino acid residues of the linear peptide backbone of the protein are, due to the unique folding, very close together in space to form
30 an epitope.

The method for the production of an antibody

The present method for the production of an antibody according to the invention comprises culturing a hybridoma cell line as mentioned above and isolating the monoclonal antibody from the supernatant of the growth media.

5 Culturing is carried out in flasks in HT- medium or in a cell culturing system called „technomouse“ in serum-free, synthetic medium (Turbodoma medium, supplied by Messi, Zurich). In a „technomouse“ hybridoma cells are cultured in a sterile chamber surrounded by a protein-impermeable membrane that is perfused by the respective medium in a constant flow rate (for example, turbomedium at 80 ml/h); antibodies are collected from the chamber
10 with the help of a syringe at regular intervals.

Isolation of monoclonal antibodies is carried out by extraction from the supernatant by conventional biochemical methods, e.g. by use of affinity columns with the corresponding immobilized antigen or by any other method used in the art, such as gel filtration or ion exchange chromatography. In the „technomouse“ supplied with serum-free
15 turbomedium antibody concentrations and purities are achieved that need no further extracting procedures.

Chimeric antibodies and fragments thereof can be produced by genetic engineering methods, e. g. by sequencing the antibody or the desired fragment thereof and constructing DNAs coding for the chimeric antibody or the fragment thereof which DNAs are inserted
20 into an appropriate expression vector and expressed to produce the antibody or the fragment thereof in both procaryotic or eukaryotic cell lines.

A fragment binding to a PrP epitope can be combined with a human heavy chain to produce chimeric antibodies for use in humans as therapeutic or preventive agents against a prion disease. A fragment binding to a PrP epitope can also be combined with other
25 enzymes, proteins or molecules to give rise to chimeric molecules combining the biological functions of these, for example for targeting an enzymatic activity to a place defined by the proximity of the PrP epitope.

The method for the production of a hybridoma cell line

30 The present method for the production of a hybridoma cell line comprises administering to PrP^{0/0} mice (knockout mice without a functional PrP gene) an immunizing amount of a recombinant pure prion protein PrP, removing the spleen from the immunized

mice, recovering splenocytes therefrom, fusing the latter with appropriate myeloma cells, growing the cells in a selection medium which does not support survival of the unfused cells, e. g. in HAT medium, screening the supernatants of the surviving hybridoma cells with recombinant PrP for the presence of antibodies to detect recombinant bovine PrP by an
5 ELISA procedure and to detect native bovine PrP^{Sc} by a conformation-sensitive ELISA procedure and isolating the positive cells. Positive hybridomas were selected and cloned twice by the limiting dilution method before the antibody was characterized and the epitope was mapped on a peptide library.

The peptide library used is commercially available from Jerini Biotools (Berlin
10 Germany). It consists of 104 spots with peptides of 13 amino acids, whereby the sequence of each peptide overlaps with 11 amino acids of the foregoing peptide.

An immunizing amount of a recombinant bovine prion protein is from about 50 to 100 µg. It is administered dissolved in an appropriate solvent, e. g. PBS and Freund's adjuvant several times, e. g. three times, subcutaneously followed by an intraperitoneal and
15 an intravenous injection ultimately prior to spleen removal.

The PrP^{0/0} mice were a gift from Prof. Weissmann of the University of Zürich. They were obtained according to Büeler et al. (1992).

Appropriate myeloma cell are for example P3X63Ag8U.1 deposited and available under ATCC CRL 1597.

20 Recovering spleen cells and fusion conditions follow standard procedures, for example as described by Kennett (1980).

The method for the production of an expression vector

25 The method for the production of an expression vector comprises inserting a DNA coding for PrP in the correct reading frame into an expression vector. One of the structures of the DNA coding for PrP is shown by SEQ ID NO:1. This DNA can be obtained by amplifying DNA from bovine genomic DNA coding for PrP by means of the N- and C-terminal primers shown by SEQ ID NO: 3 and SEQ ID NO: 4., respectively. Bovine
30 genomic DNA is isolated from bovine kidney cells and supplied by Clontech, U.S.A. Degenerate allelic forms of this DNA coding for the same PrP may be used. Furthermore,

targeted mutations can be introduced into the PrP DNA to give rise to distinct conformational isoforms of the translated gene product.

The production of a purified recombinant bovine PrP

5 The production of a purified recombinant bovine PrP comprises culturing a cell line with an expression vector capable of expressing the bovine PrP in an appropriate culture medium, such as in the case of E. coli in Luria broth medium, isolating the PrP protein from the inclusion bodies by lysing the cells e. g. with lysozym and Triton-X-100 in the case of E. coli, solubilizing the inclusion bodies with urea and and purifying the protein. by
10 conventional methods, e. g. by chromatography, for example on a anionic exchange sepharose column and C4 reverse phase HPLC column.

 The oxidized form is obtained by treament with an oxidizing agent, e. g. with Cu_2SO_4 , and the reduced form by treatment with a reducing agent, e. g. β -mercaptoethanol, according ot conventional methods. They can be separated by reverse-phase high pressure
15 liquid chromatography.

Immunological detection procedure for the detection of prion disease

20 An immunological detection procedure for the detection of prion disease, especially BSE, whereby disease-specific PrP^{Sc} protein in biological material of an animal or human is detected, comprises treatment of a first probe of said material with a monoclonal antibody according to the invention and detecting the mixed $\text{PrP}^{\text{C}}/\text{PrP}^{\text{Sc}}$ - antibody complex, treating a second probe of said material first with proteinase K and then with the monoclonal
25 antibody according to the invention, detecting the PrP^{Sc} -antibody complex and analyzing the results of both probes.

 A specific monoclonal antibody according to the invention is able to detect PrP^{Sc} in a PrP^{Sc} -antibody complex without prior protease-digestion of the tissue specimen to be examined.

30 The biological material can be insoluble or soluble in buffer or body fluids. It can be derived from any part of the body, e. g. from the brain or the tissue sections, in which case it is used in form of a homogenate, or any body fluid, e. g. cerebrospinal fluid, urine, saliva

or blood. In the case of body fluids, fluid-resident cells, e.g. white blood cells in the case of blood expressing PrP can be purified and analyzed either in immunohistochemistry or as a homogenate.

The detection of the PrP^{Sc}-antibody complex is carried out in particular by immunological procedures like the Western blotting, ELIFA, and various ELISA techniques such as capture ELISA.

The present immunological detection procedures allow the diagnosis of prion diseases. With the tools of the present invention, tissue sections, tissue homogenates or body fluids of prion-infected animals such as BSE-diseased cattle or humans having the CJD can be screened for the presence of the protease-resistant, disease-specific isoform of the prion protein in its native form, be it soluble or insoluble.

Tissue homogenates and body fluids are for example such as from biopsy of brain, lymph nodes, spleens, tonsils, peripheral nerves, cerebrospinal fluids, urine, platelets or white blood cells. Particular immunological procedures comprise for example, enzyme-linked immunofiltration assay (ELIFA), enzyme-linked immunoabsorbent assay (ELISA), Western blot assay, dot blot assay, immunodecoration and immunohistochemistry.

When native bovine PrP^{Sc} or any other disease-specific prion protein (e.g. ovine PrP^{Sc} or human PrP^{Sc}) has to be used in immunological assays, this can presently only successfully be achieved with the antibodies described in the present invention, since the present antibodies are the first of their art to be able to bind only native, disease-specific PrP^{Sc}.

The test kit for the diagnosis of prion diseases

25

The test kit for the diagnosis of prion diseases comprises devices and materials enabling the diagnosis prion disease in biological materials, and is particularly suited for screening large amounts of samples for the presence of PrP^{Sc}. One test kit comprises in particular one or more monoclonal antibodies according to the invention, purified bovine recombinant PrP protein as mentioned above, nitrocellulose sheets, microtiter plates, or microtiter plates coated with the monoclonal antibodies according to the invention, a secondary anti-mouse antibody that is coupled with an enzyme and its substrate or any other

molecular compound for a detection reaction (e.g. a peroxidase-labeled anti-mouse IgG antibody, TMB or any other peroxidase substrate), hydrogen peroxide, proteinase K, a blocking buffer, a homogenization buffer, a calibration curve and a description of how to perform the test.

5

Another test kit is designed in the dipstick format and is without need of radioactive tracers, enzymes or substrates and basically reduces the number of handling steps to one. The one-step procedure involves the capture of the disease-specific PrP^{Sc} with one of the antibodies according to claim 1 or 2 which are immobilized on a test strip. Captured
10 disease-specific PrP^{Sc} are detected directly by a second antibody according to the invention, which is coupled to particular colloid particles. This specific detector complex results in the formation of coloured spots on the test strip which are visible in less than 30 minutes depending on the concentration of the test sample. The spots are a permanent record of the test result and, upon longer exposure even increase the sensitivity of the test
15 without generating higher background.

Pharmaceutical preparation for the therapy and prevention of prion diseases

The pharmaceutical preparation for the therapy and prevention of prion diseases in a
20 mammal, including humans, comprises an effective amount of one or more antibodies fragments thereof or chimeric antibodies as described, produced according to the invention, eventually purified according to conventional methods, and a conventional pharmaceutical carrier. An antibody obtained may be solubilized together with the carrier in an appropriate buffer, e. g. an aqueous physiological sodium chloride solution. This may be clarified by
25 centrifugation and used in concentrated liquid form for injection, or completely dried if desired by any of the conventional methods, such as lyophilization, spray or freeze drying, in form of a dry powder, which can be pressed into tablets, filled into capsules, or applied as a dry powder in form of a nasal spray, whereby conventional production methods are applied, and conventional pharmaceutical carriers are optionally added.

30

Method of protecting a mammal against prion disease

The monoclonal antibodies of the present invention bind to between species highly conserved regions in the PrP molecule that may have functional significance (Oesch et al., 1991). It is envisioned that blocking this binding site by the monoclonal antibodies, fragments thereof or chimeric antibodies as defined above will abolish biological effects of prions. Blocking of the infectivity of prions by occupying distinct sites on the disease-specific form of PrP is foreseen to represent a therapeutic strategy in treating prion diseases or a preventive strategy in preincubating suspected prion-infected tissue specimens with the present monoclonal antibodies. The normal form of PrP appears not to be of vital importance in the living animal because mice with a deleted PrP are viable (Bueler et al., 1992). Anti-PrP antibodies may therefore be used without side effects to neutralize prions in humans or animals.

The present invention concerns further a method for the therapy or prevention of prion disease or a disease mediated by the neurotoxic effects of prion proteins or fragments of prion proteins, comprising administering to a patient suffering from such disease or being likely to becoming a victim of this disease a therapeutical or preventive amount of a monoclonal antibody, a fragment thereof or a chimeric antibody as described above.

The method of protecting a mammal, including a human, against an infectious prion disease according to the present invention comprises administering one or a combination of the present antibodies, fragments thereof or chimeric antibodies or a pharmaceutical preparation comprising the antibodies produced by the present invention. The pharmaceutical preparation is preferably administered by injection, e. g. intrathecally (into the cerebrospinal fluid), into the blood with respective pharmaceutical agents or methods increasing the permeability of the blood-brain barrier or as a chimeric antibody, fused to, or containing additional signal sequences that allow passage through the blood-brain barrier (for review see Friden, 1994). An intranasal application of the monoclonal antibodies, fragments or chimeras thereof is also possible.

The pharmaceutical preparations have to be administered according to the judgment of the physician in amounts depending on the concentration of the antibodies comprised thereby and the route of administration so that a protective or curative effect is obtained. The amounts and method of administration are to be selected further depending upon the age and weight of the patient, the nature and severity of the infection as well as the general

condition of the patient. In general it is sufficient to administer the antibodies in amounts of about 1 to 100 mg per patient in a single or in repeated doses.

Method for clearing biological material from prions

5 The method for clearing biological material from prions, e. g. intended for transplantation, substitution of biological material or oral consumption, comprises treating said material with one or several monoclonal antibodies according to the invention such that prions or prion proteins or fragments thereof become functionally inactivated in terms of their infectivity and/or neurotoxicity. The pharmaceutical preparations described above may
10 be used for this purpose, whereby the pharmaceutical carrier may be replaced by a suitable other solvent in case the biological material is not intended to be used for transplantation.

 The following examples serve to illustrate a particular embodiment of the invention but they should not be considered a limitation thereof. The immunological procedures outlined are made for the diagnosis of BSE in cattle, however, these procedures can also be
15 applied for prion diseases in humans or animals such as sheep, hamsters or mice.

Method for protecting an animal or a human by immunization with recombinant PrP

 The method for protecting animals or humans from infection with prions consists of an appropriate formulation of recombinant PrP of the appropriate species with an
20 immunostimulator such as Freund's adjuvans. To protect against BSE, immunization is done with bovine PrP, against CJD human PrP is used, against sheep scrapie sheep PrP is used. In general, the PrP of the species where the prion originated is used for immunization. Immunization induces the β -lymphocytes to produce antibodies reacting with PrP^{Sc}. Such antibodies (of which 15B3 is a prototype) will be present in blood and lymphatic tissue and
25 thereby bind and neutralize prions infecting a human or animal through peripheral pathways such as through skin lesions for example after accidental puncture with a needle or knife in a hospital or a slaughterhouse. The amount and type of PrP to be used for immunization will be determined according to the age and weight of the human or animal as well as the source of prions.

30

Example 1. Immunological diagnosis of prion diseases using tissue from infected animals

PrP^C and PrP^{Sc} can be distinguished according to their different sensitivity to digestion with protease K. Undigested PrP^C and PrP^{Sc} have a molecular weight of 33-35 kDa. Upon incubation with proteinase K PrP^C is readily digested while PrP^{Sc} is partially resistant, i.e. the N-terminus of PrP^{Sc} is removed leading to a shift in molecular weight from 33-35 kDa to 27-30 kD (Oesch et al., 1985). Proteinase K is therefore used to digest the tissue specimen to be examined. However, monoclonal antibody 15B3, detecting a conformational epitope specific for the disease-specific isoform PrP^{Sc} may even be used without prior protease digestion.

Example 1.1. Method for the preparation of tissue homogenates

One gram of brain, either from the thalamus, medulla or spinal cord, were homogenized with an homogenizer (Omni, USA) in 10 ml 10% sucrose, 20 mM HEPES pH 7.5, 2% sarcosyl and 5 mM EDTA. 10% homogenates were diluted 10 fold; one part of the homogenate was digested with proteinase K at 0, 10 or 100 µg/ml. The probe containing 0 µg/ml served as a control for the specificity of the proteinase K treatment. Probes were then further diluted (in PBS) for the ELIFA test to give a blotting concentration of 0.05% of brain homogenate. To increase the partial protease resistance of bovine PrP^{Sc}, brain homogenates from BSE-infected and normal cattle was diluted after homogenization in 20, 40 or 80% ethanol /HEPES-sucrose buffer. Suspension of brain homogenates in ethanol was an important step and effectively stabilized the β-sheet structure of the PrP^{Sc} isoform (Oesch et al., 1994), thereby increasing its protease resistance (Riesner et al., 1996).

Example 1.2. Western Blotting

Tissue specimens were homogenized as described in example 1.1., one part protease-digested, the other not (as described above), diluted to 10% and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels (Sambrook et al., 1989).

Gels were then electroblotted onto 0.45 µm nitrocellulose (NC) membranes, incubated with the respective monoclonal antibodies followed by a secondary anti-mouse IgG antibody coupled to peroxidase. Bound peroxidase activity was detected with a chemiluminescence kit (ECL, Amersham, USA).

5 Western blots developed with antibodies 6H4 or 34C9 (Figure 1A.) show the characteristic smear of bands for PrP^C and bovine PrP^{Sc} (33 to 27 kD) in undigested probes while digestion with proteinase K eliminates all of PrP^C, however, leaving a 27 kD band typical of N-terminally truncated PrP^{Sc}. The smear is due to different glycosylated forms of PrP.

10 The present antibodies were furthermore able to detect PrP in various tissue extracts from humans, cattle, pigs, sheep, mice and hamsters (Figures 1B., 2). Given that the epitopes of our antibodies are highly conserved (Oesch et al., 1991), we expect our antibodies to stain PrP from other species such as rat or marsupials as well.

15

Example 1.3. ELIFA (Enzyme Linked Immuno Filtration Assay)

The ELIFA procedure to determine quantitatively the amounts of PrP^C and PrP^{Sc} in given tissue homogenates has been described for hamster brain homogenates (Oesch et al., 1994). Blotting directly on nitrocellulose has the advantage over the conventional
20 ELISA procedure that the poor solubility of bovine PrP^{Sc} does not affect its immobilization on the solid phase. Blotting was performed with an ELIFA apparatus (Pierce), i.e. a peristaltic pump created a vacuum below the NC thereby sucking the contents of the wells above onto the NC in a controlled and highly reproducible way. Wells were then washed
25 with PBS. The membrane was removed from the ELIFA apparatus, placed in a plastic tray and then incubated on a rocking table sequentially for the indicated times with the following reagents (inbetween steps, the filters were always washed 3x with PBS): 5% BSA/TBST (30min); avidin (25 µg/ml, 30min); biotin (2 µg/ml; 30min); monoclonal antibodies 6H4 or 34C9 in TBST (2 h RT or 10 h o/n); secondary, biotinylated anti-mouse IgG (Vectastain,
30 USA, dilution 1:5000; 1 h RT); streptavidin coupled to peroxidase (Boehringer, Germany, dilution 1:25000; 15-60 min RT).

In an alternative procedure, monoclonal antibodies 6H4 or 34C9 were biotinylated eliminating the step with the biotinylated secondary antibody. Still another procedure involved coupling of mABs 6H4 or 34C9 directly to peroxidase according to the manufacturer (Pierce, USA). Amplification of peroxidase activity was achieved by the
5 ELAST-kit according to the manufacturer (DuPont, USA).

Subsequently, the NC was again placed in the ELIFA apparatus, a 96-well microtiterplate was placed underneath the membrane, such that each blotted spot corresponded to one well in that plate. Then the substrate TMB/peroxide (Kierkegaard & Perry) was applied into the wells of the ELIFA apparatus and sucked through the membrane
10 into the wells of the microtiter plate. The reaction was stopped by the addition of 2M H_3PO_4 . The extinction was measured at 450 nm with a reference at 620 nm in an ELISA reader.

The standard curve for the ELIFA (see Figure 3a) was obtained by serial dilutions of ultra-pure and defined amounts of recombinant bovine PrP (see below). For the ELIFA-
15 procedure, lyophilized recombinant PrP was suspended in an antigen-dilution buffer (1M guanidinium thiocyanate and 0.01% human serum albumin in PBS). This buffer allows maximum binding of recombinant PrP to the nitrocellulose membrane. The standard curve is essential, since it allows to control both the quality and the reliability of the ELIFA-procedure. Furthermore, the standard curve allows to exactly quantify bovine PrP^C/PrP^{Sc}
20 amounts in given tissue specimens (Oesch et al., 1994) (Figure 3b).

Example 1.4. Conventional ELISA (Enzyme Linked Immuno Sorbent Assay)

The antigen (present in a 10% homogenate as described in example 1.1.) was
25 incubated for 2 h at RT in 96-well microtiterplates (Nunc, Denmark). Blocking was achieved with 5% BSA after antigen incubation. After washing, the plate was incubated with the biotinylated monoclonal antibody or 6H4 for 2 h at RT. Washing with H_2O and PBS was performed before streptavidin-coupled peroxidase (Boehringer, Germany) was applied and peroxidase activity detected with the substrate according to the manufacturer
30 (TMB/ H_2O_2 ; Kierkegaard & Perry). The reaction was stopped by the addition of 2 M H_3PO_4 . As for the ELIFA procedure, the plate is read by an ELISA reader at 450 nm with a reference at 620 nm. As an alternative procedure, peroxidase-coupled monoclonal antibody

6H4 was used instead of biotinylated antibody (see example 1.3.). Amplification of peroxidase activity was achieved by the ELAST-kit according to the manufacturer (DuPont, USA). Results of this immunological test are depicted in figure 3c.

5 **Example 1.5. Capture-ELISA (Enzyme Linked Immuno Sorbent Assay)**

For the capture-ELISA, advantage can be taken of the multimeric nature of disease-specific PrP^{Sc}. Using the same monoclonal antibody for coating the wells and detection of native PrP (e.g. 6H4) will detect only PrP that exists in multimeric form or aggregated states, since monomeric PrP will have blocked its single binding site for the detection mAB by the coating mAB. Multimeric PrP^{Sc} will be detected because apart from the one mAB binding site that couples the multimeric PrP to the microtiter plate, other binding sites are still present for the detection antibodies (see Figure 4). This particular procedure of a capture-ELISA for PrP^{Sc} detection can only be performed with the present monoclonal antibodies binding to native PrP^{Sc}, since upon denaturation the multimeric PrP^{Sc} complexes dissociate into multiple monomeric, denatured PrP molecules.

For the capture ELISA, monoclonal antibodies according to the invention were either covalently linked or adsorbed to microtiterplates (Nunc, Denmark). Subsequently, the wells were blocked with 5% BSA for 30 min at RT and the procedure according to example 1.4. followed.

Examples 2. Experimental details for the production of monoclonal antibodies specific for the native and denatured prion protein

25

Example 2.1. Preparation of the immunogen

The following primers were chosen to amplify PrP DNA from bovine genomic DNA:

30

1. N-terminal sense primer (SEQ ID NO: 3):

5'-GGGAATTCCATATGAAGAAGCGACCAAAACCTG-3' and

2. C-terminal antisense primer (SEQ ID NO4):

5'-CGGGATCCTATTAAGTTGCCCCCTCGTTGGTA-3'.

These primers were designed to introduce an Nde I restriction site at the 5' end and a BamH I restriction site at the 3' end in the PCR-amplified bovine PrP-DNA. 10 cycles of 1 min 94°C, 2min 42°C and 2min 72°C, followed by 4x5 cycles of 1min 94°C, 1min 63°C and 72°C with augmenting durations of 1,2,3 and 4 min were performed in a Crocodile III thermocycler (Appligene, USA).

The PCR product then digested with the restriction enzymes Nde I and BamH I (Boehringer, Germany). The appropriate DNA fragment of 650 bp was purified on a 1% agarose gel and ligated into the pET11a vector (Novagen) previously digested with Nde I and BamHI. Ligated products were transfected into E. coli strain DH5α. The appropriate clone containing the PrP open reading frame in the pET11a vector was selected and subsequently termed pbPrP3 (Figure 5). Sequencing of the PrP sequence in pbPrP3 confirmed that the sequence of the bovine PrP gene in the pET11a vector corresponds to the previously published sequence (Goldmann et al., 1991; SEQ ID NO: 1). Plasmid pbPrP3 was transfected into E. coli strain BL21(DE3) (Novagen), that is capable of translating the plasmid into a protein. For production of bovine PrP, cells were stimulated with 1 mM IPTG according to standard techniques (Sambrook et al., 1989). Recombinant bovine PrP was purified from inclusion bodies. For example, 1 liter of Luria broth medium (Gibco, USA) containing 100 µg/ml ampicillin was incubated with an overnight culture (4ml) of pbPrP3 transfected BL21(DE3) cells. The 1l culture was then grown at 37°C and 250 rpm to an OD₆₀₀ of 0.8. IPTG was added to a final concentration of 1 mM and the incubation was continued at 30°C and 250 rpm for 3h until the OD₆₀₀ was 1.0. The culture was then centrifuged at 1000 x g for 5 min at RT. The pellet containing the bacteria was further processed for isolation of PrP from inclusion bodies as follows: the bacterial pellet from a 1 liter culture was resuspended in 100 ml of 2 mM EDTA, 50 mMTris-HCl pH 7.5 and lysed by the addition of lysozyme (final concentration 100 mg/ml) and Triton-X-100 (final conc. 1%) for 15 min at 37°C. Then, MgCl₂ (final conc. 15 mM) and DNase I (final conc. 10 µg/ml) were added. The suspension was shaken at room temperature until the DNA was digested and the solution was clear (30 min). The solution was then centrifuged at 10.000 x g for 30 min at 4°C. The supernatant containing all the soluble proteins was discarded and the pellet, containing the inclusion bodies, was homogenized and resuspended in 1/10th of the original culture volume containing 8M deionized urea, 10 mM MOPS pH 7.5. This

suspension was then shaken at room temperature overnight and then centrifuged at 10.000 x g for 30 min at 4°C. The supernatant containing solubilized material from inclusion bodies was then further processed:

5 A carboxymethyl (CM) sepharose column (Pharmacia) was equilibrated first with the elution buffer containing 8M deionized urea, 500 mM NaCl, 10mM MOPS pH 7.5, then with the washing buffer containing 8M deionized urea, 10mM MOPS pH 7. A CM sepharose column (50 ml bed volume) was loaded with 100 ml of the solution containing the solubilized proteins from inclusion bodies. The column was washed twice with 25 ml 50
10 mM NaCl, 8M urea, 10 mM MOPS and once with 100 mM NaCl, 8M urea, 10 mM MOPS. Bovine recombinant PrP was eluted with 500 mM NaCl, 8M urea, 10 mM MOPS. SDS-PAGE and silver staining showed that at this step only one protein of about 24 kDa was present in the eluent, corresponding to the calculated molecular weight of 23,6 kDa (Figure 6a). This fraction was then further processed.

15 Proteins eluted from the CM sepharose were subsequently either oxidized with 10 µM Cu₂SO₄ or reduced with 2% β-mercaptoethanol for several hours before they were loaded on a C₄-reverse phase HPLC column. The HPLC column was perfused with a 0-85% gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). The oxidized or reduced
20 bovine recombinant PrP eluted about 40 or 45% acetonitrile, respectively. The eluted fractions were lyophilized (Sambrook et al., 1989) and redissolved in distilled water. Electrospray mass spectroscopy revealed single peaks of 23676.8 and 23686.1 Dalton for oxidized and reduced recombinant bovinePrP, respectively, indicating a correct and uniform translation of the bovine PrP open reading frame in pbPrP3.

25

Example 2.2: Immunization of animals and hybridoma production

Oxidized or reduced bovine recombinant PrP or a mixture of both amounting to a total of 100 µg in a single dose (dissolved in PBS) were used to immunize PrP^{0/0} mice, i.e.
30 mice without a functional PrP gene (Bueler et al., 1992) that were kindly provided by Prof. C. Weissmann, University of Zurich. The reduced form of the recombinant PrP was particularly interesting since it has been reported to contain more β-sheet secondary

structures than the oxidized form in a Syrian hamster recombinant PrP fragment (Mehlhorn et al., 1996), hence mimicking structural features of PrP^{Sc}, however, the reduced recombinant isoforms have been reported to be neither protease-resistant nor infectious (Mehlhorn et al., 1996).

5 Mice received three subcutaneous injections (day 0 with Freund's complete adjuvans, days 21 and 42 with Freund's incomplete adjuvans) of the antigens in a constant 100µg amount and in a volume of 100µl. On day 49, mice were boosted with the antigen intraperitoneally and the next day intravenously with adjuvant Pertussi Berna (Berna Switzerland; extract of Bordetella pertussis bacteria). On day 50, mice were anesthetized
10 and decapitated. The spleen from immunized mice was removed, and splenocytes were recovered. Mouse myeloma cells (cell line P3X63Ag8U.1, ATCC CRL 1597; Scharff, 1978) were mixed to the splenocytes at a ratio of 1:5 and fused by the addition of 50% PEG (polyethylenglycol) for 8 min at RT according to standard techniques (Kennett, 1980). Cells were then washed and grown overnight. The next day, cells were suspended in selective
15 medium (HAT) and plated in 96-well microtiterplates. The selective medium contains aminopterin that is toxic for those cells that have not been fused to splenocytes and thus eliminates uncontrolled cell growth of irrelevant cells (Kennett, 1980).

EXAMPLE 2.3: Screening hybridomas for specific antibodies

20 Most important was an efficient screening method for antibody-producing hybridoma cell lines that would allow to detect monoclonal antibodies against native and denatured epitopes of both PrP isoforms, as well as conformation-specific epitopes of bovine PrP^{Sc}. The screening for hybridoma cells producing antibodies against PrP was done by an ELISA, Western blotting and a conformation-sensitive ELIFA.

25

ELISA using recombinant bovine PrP

96-well microtiter plates were coated with recombinant bovine PrP (0.25 µg/well) for 4 h at RT and then blocked with 5% BSA/H₂O for 1h at RT. After washing with H₂O and PBS, culture medium from wells containing hybridoma colonies was transferred to the
30 microtiter plates (50 µl per well) and incubated overnight at 4°C. After washing with H₂O and PBS, bound antibodies were detected with a peroxidase-labeled anti- mouse IgG

antibody (Cappell, Switzerland) followed by colorimetric detection with TMB/H₂O₂ (Kierkegaard & Perry, USA) as described in example 1.4.

Qualitative, conformation-sensitive ELIFA

5 1% brain homogenates of normal and BSE-infected cattle were either left undigested or protease-digested for the BSE brain homogenate and blotted onto a nitrocellulose membrane as described by for the ELIFA procedure (see above) (Oesch et al., 1994). After blotting, the membrane was blocked with 5% low-fat milk in TBST, and incubated with the antibody-containing culture medium. Subsequently, the NC was
10 incubated with a secondary, peroxidase-coupled anti-mouse IgG antibody and developed with a chemiluminescence kit (ECL, Amersham). This technique allowed for detection of hybridoma cell lines that produce antibodies against native PrP^C and bovine PrP^{Sc} or of conformation-sensitive antibodies that distinguish between PrP^C and bovine PrP^{Sc}(as described for mAB 15B3 in figure 6b).

15

Western Blotting

Hybridoma cell lines were further selected on the capability of the produced antibodies to recognize PrP of brain homogenates and recombinant bovine PrP on Western blots. Brain homogenates of various tissues and various species were blotted as described in
20 example 1.2..

It was shown that the preferred monoclonal antibody 6H4 recognizes PrP in the brain homogenates of cattle, mice, hamsters, pig, sheep and humans (Figure 2).

The preferred mAB 34C9 recognizes PrP in the brain homogenates of cattle, mice, pig, and humans (Figure 2).

25 It was further shown that both preferred monoclonal antibodies 6H4 and 34C9 recognize PrP in various tissues such as medulla, spinal cord, thalamus, cortex and white blood cells (Figures 1a, b).

Mapping of epitopes

30 A peptide library consisting of 104 peptides numbered 1 to 104 purchased from Jerini Biotoools (Berlin, Germany) was used to map the epitopes that are recognized by the antibodies. The peptides are covalently linked to a cellulose membrane have each a length of

13 amino acids and together cover the entire length of the recombinant bovine prion protein (total of 104); starting with Lys Lys Arg Pro Lys Pro Gly Gly Gly Trp Asn Thr Gly (one letter code: KKRPKPGGGWNTG) and ending with Gln Arg Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ala Ser (one letter code: QRESQAYYQRGAS). Each peptide overlaps by 11 amino acids with the next peptide. Binding of antibodies to those peptides can be visualized by the ECL system as described for Western blotting in example 1.2..

The monoclonal antibodies of the present invention bound to peptides comprised in the region of helix 1 in the three-dimensional model of the mouse recombinant C-terminal prion protein fragment described by Riek et al. (1996). It is hereby assumed that this mouse recombinant C-terminal prion protein fragment reflects structures of native PrP^C, has the same structure as full length PrP and that the structure will be similar in different species. Based on these assumptions the following statements are: mAB 6H4 binds to the three library peptides Nos. 64 to 66, comprising amino acids 155-163 of the bovine PrP sequence (Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu; Goldmann et al., 1991) (Figure 7b). This sequence corresponds exactly to the full-length helix, a structure that is highly conserved between species (Oesch et al., 1991).

mAB 34C9 binds to the 5 library peptides Nos. 59 to 63 comprising amino acids 149-153 of the bovine PrP sequence (Leu Ile His Phe Gly; Goldmann et al., 1991) (Figure 7a) which corresponds to a sequence just N-terminal of helix 1 (Riek et al., 1996).

As predicted by this epitope mapping, the monoclonal antibodies differentially bind PrP from different species (Figure 2).

Characterization of PrP^{Sc} conformation-specific monoclonal antibody 15B3

mAB 15B3 recognizes 3 distinct arrays of peptides: Nos. 62 to 65 of the peptide library comprising amino acids 153 to 159 (Gly Ser Asp Tyr Glu Asp Arg), Nos. 73 to 75 comprising amino acids 173 to 181 (Tyr Tyr Arg Pro Val Asp Gln Tyr Ser) and No. 102 comprising amino acids 225 to 237 (Cys Ile Thr Gln Tyr Gln Arg Glu Ser Gln Ala Tyr Tyr) of bovine PrP according to Goldmann et al. (1991).

Monoclonal antibody 15B3 recognizes native bovine PrP^{Sc} better than native bovine PrP^C (Figure 6b). In this experiment, 10% bovine brain homogenates of normal undigested and BSE-diseased protease-digested cattle were made as described in example 1.1.. Subsequently, the samples were diluted to a 0.5% homogenate with PBS and

incubated at 37°C for 1h. The samples were then blotted onto a nitrocellulose membrane with the qualitative, conformation-sensitive ELIFA protocol as described in example 3 above. In Figure 6b, it can be seen that 15B3 binds to BSE but not to normal brain homogenate. By Western blotting, PrP from bovine brain homogenates cannot be detected (Figure 6a). Apparently mAB 15B3 cannot detect PrP on Western blotting even if it is assumed that proteins denature in sample buffer containing SDS before they are loaded on the gel (Example 1.2.; Sambrook et al. 1989).

These findings point to the fact that mAB 15B3 binds to a conformation-sensitive epitope. As can be seen in the binding experiments with the peptide library, mAB 15B3 binds to several distant peptides as would be expected for a conformation-sensitive mAB.

The specificity of the antibody 15B3 was further confirmed by immunoprecipitation (Figure 8). While 6H4 precipitated PrP from normal as well as PrP^{Sc}-containing homogenates, 15B3 precipitated only PrP^{Sc} from infected cattle, mice or humans (Figure 1A, C, D, respectively). The precipitated PrP was shown to be protease resistant (Figure 1B, C,D). For immunoprecipitation, 200 µl 1 % brain homogenates were incubated for 2 h at room temperature with 200 µl 0,25 µg/ml antibody-containing serum-free medium; after incubation with additional 50 µl protein A- or protein G-coupled agarose (for 15B3 and 6H4, respectively; Boehringer Mannheim) for 2 h at room temperature, agarose beads were centrifuged and the pellet washed according to the manufacturer. Pelleted proteins were analyzed on Western blots.

Example 3: Reduction of infectivity of prions by monoclonal antibodies

Brain homogenates from BSE-infected cattle are obtained as described in example 1.1. The exact amount of present bovine PrP^{Sc} are measured with the help of the ELIFA technique or the ELISA technique as described in examples 1.3 to 1.5, respectively. Serial dilutions of this infected brain homogenate are aliquoted. To these serial dilutions are added the preferred mABs 6H4 or 34C9 or 15B3, or a mixture thereof, in molar amounts exceeding the molar amounts of measured PrP^{Sc}. The mix is incubated for 4h at RT and then 100 µl are injected intracerebrally into the animal. Transgenic mice overexpressing mouse PrP (tg35; Fischer et al., 1996) are used as an animal model for measuring the infectivity of bovine PrP^{Sc}.

List of buffers and solutions

HT-medium	450 ml Iscove's modified Dulbecco's medium (GIBCO) 30 ml sterile human serum 5 ml glutamine (200 mM) 5 ml hypoxanthine (10 mM)/thymidine (1.5 mM) 5 ml penicillin (10000 IU/ml)/streptomycin (10000µg/ml) 250 µl sterile β-mercaptoethanol
HAT-medium	HT-medium + 2mM aminopterin
TBS	20 mM Tris pH 7.5 150 mM NaCl 0.05% Tween 20
TBST	20 mM Tris pH 7,5 150 mM NaCl 0.05% Tween 20

5 Deposit of Microorganisms

The hybridoma cell lines were deposited under the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-381324 Braunschweig, as follows:

1. Hybridoma cell line producing mAB 34C9: DSM ACC2295, deposited February 06,
10 1997
2. Hybridoma cell line producing mAB 6H4: DSM ACC2296, deposited February 06,
1997
3. Hybridoma cell line producing mAB 15B3: DSM ACC2298, deposited February 13,
1997

15

References

- Alper, T., Haig, D.A., and Clarke, M.C. (1978). The scrapie agent: evidence against its dependence for replication on intrinsic nucleic acid. *J. Gen. Virol.* *41*, 503-516.
- 5 Anderson, R.M., Donnelly, C.A., Ferguson, N.M., Woolhouse, M.E.J., Watt, C.J., Udy, H.J., MaWinney, S., Dunstan, S.P., Southwood, T.R.E., Wilesmith, J.W., Ryan, J.B.M., Hoinville, L.J., Hillerton, J.E., Austin, A.R., and Wells, G.A.H. (1996). Transmission dynamics and epidemiology of BSE in british cattle. *Nature* *382*, 779-788.
- 10 Barry, R.A., Kent, S.B., McKinley, M.P., Meyer, R.K., DeArmond, S.J., Hood, L.E., and Prusiner, S.B. (1986). Scrapie and cellular prion proteins share polypeptide epitopes. *J. Infect. Dis.* *153*, 848-854.
- 15 Barry, R.A. and Prusiner, S.B. (1986). Monoclonal antibodies to the cellular and scrapie prion proteins. *J. Infect. Dis.* *154*, 518-521.
- Basler, K., Oesch, B., Scott, M., Westaway, D., Walchli, M., Groth, D.F., McKinley, M.P., Prusiner, S.B., and Weissmann, C. (1986). Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* *46*, 417-428.
- 20 Bessen, R.A., Kocisko, D.A., Raymond, G.J., Nandan, S., Lansbury, P.T., and Caughey, B. (1995). Non-genetic propagation of strain-specific properties of scrapie prion protein (see comments). *Nature* *375*, 698-700.
- 25 Bolton, D.C., McKinley, M.P., and Prusiner, S.B. (1982). Identification of a protein that purifies with the scrapie prion. *Science* *218*, 1309-1311.
- Bruce, M., Chree, A., McConnell, I., Foster, J., Pearson, G., and Fraser, H. (1994). Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* *343*, 405-411.
- 30 Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M., and Weissmann, C. (1992). Normal development and behaviour of mice lacking the neuronal cell- surface PrP protein (see comments). *Nature* *356*, 577-582.
- 35 Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M., and Weissmann, C. (1993). Mice devoid of PrP are resistant to scrapie. *Cell* *73*, 1339-1347.
- 40 Cohen, F.E., Pan, K.M., Huang, Z., Baldwin, M., Fletterick, R.J., and Prusiner, S.B. (1994). Structural clues to prion replication. *Science* *264*, 530-531.
- Collinge, J., Whittington, M.A., Sidle, K.C., Smith, C.J., Palmer, M.S., Clarke, A.R., and Jefferys, J.G. (1994). Prion protein is necessary for normal synaptic function. *Nature* *370*, 295-297.
- 45

- Collinge, J., Sidle, K.C.L., Meads, J., Ironside, J., and Hill, A.F. (1996). Molecular analysis of prion strain variation and the etiology of a "new variant" CJD. *Nature* 383, 683-690.
- 5 Diener, T.O., McKinley, M.P., and Prusiner, S.B. (1982). Viroids and prions. *Proc. Natl. Acad. Sci. U. S. A.* 79, 5220-5224.
- Friden, P.M. (1994). Receptor-mediated transport of therapeutics across the blood-brain barrier. *Neurosurgery* 35, 294-298. (Abstract)
- 10 Garfin, D.E., Stites, D.P., Perlman, J.D., Cochran, S.P., and Prusiner, S.B. (1978). Mitogen stimulation of splenocytes from mice infected with scrapie agent. *J. Infect. Dis.* 138, 396-400.
- 15 Goldmann, W., Hunter, N., Martin, T., Dawson, M., and Hope, J. Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon. *J. Gen Virol.* 72:201-204, 1991
- Griffith, J.S. (1967). Self-replication and scrapie. *Nature* 215, 1043-1044.
- 20 Hecker, R., Taraboulos, A., Scott, M., Pan, K.M., Yang, S.L., Torchia, M., Jendroska, K., DeArmond, S.J., and Prusiner, S.B. (1992). Replication of distinct scrapie prion isolates is region specific in brains of transgenic mice and hamsters. *Genes Dev.* 6, 1213-1228.
- 25 Hope, J., Reekie, L.J., Hunter, N., Multhaup, G., Beyreuther, K., White, H., Scott, A.C., Stack, M.J., Dawson, M., and Wells, G.A. (1988). Fibrils from brains of cows with new cattle disease contain scrapie- associated protein. *Nature* 336, 390-392.
- 30 Jendroska, K., Heinzel, F.P., Torchia, M., Stowring, L., Kretzschmar, H.A., Kon, A., Stern, A., Prusiner, S.B., and DeArmond, S.J. (1991). Proteinase-resistant prion protein accumulation in Syrian hamster brain correlates with regional pathology and scrapie infectivity. *Neurology* 41, 1482-1490.
- 35 Kascak, R.J., Rubenstein, R., Merz, P.A., Tonna DeMasi, M., Fersko, R., Carp, R.I., Wisniewski, H.M., and Diringer, H. (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J. Virol.* 61, 3688-3693.
- 40 Kasper, K.C., Stites, D.P., Bowman, K.A., Panitch, H., and Prusiner, S.B. (1982). Immunological studies of scrapie infection. *J. Neuroimmunol.* 3, 187-201.
- Kennett, R.H. (1980). Fusion centriugation of cells suspended in polyethylene glycol. In *Monoclonal antibodies. Hybridomas: a new dimension in biological analysis.* R.H. Kennett, T.J. McKearn, and K.B. Bechtol, eds. (New York: Plenum Press), pp. 365-367.
- 45 Kocisko, D.A., Come, J.H., Priola, S.A., Chesebro, B., Raymond, G.J., Lansbury, P.T., and Caughey, B. (1994). Cell-free formation of protease-resistant prion protein. *Nature* 370, 471-474.

- Krasemann, S., Groschup, M., Harmeyer, S., Hunsmann, G., and Bodemer, W. (1996). Generation of monoclonal antibodies against human prion proteins in PrP^{0/0}-mice. *Molecular Medicine* 2, 725-734.
- 5 Mehlhorn, I., Groth, D., Stockel, J., Moffat, B., Reilly, D., Yansura, D., Willett, W.S., Baldwin, M., Fletterick, R., Cohen, F.E., Vandlen, R., Henner, D., and Prusiner, S.B. (1996). High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. *Biochemistry* 35, 5528-5537.
- 10 Oesch, B., Westaway, D., and Prusiner, S.B. (1991). Prion protein genes: evolutionary and functional aspects. *Curr. Top. Microbiol. Immunol.* 172, 109-124.
- Oesch, B., Westaway, D., Walchli, M., McKinley, M.P., Kent, S.B., Aebersold, R., Barry, R.A., Tempst, P., Teplow, D.B., and Hood, L.E. (1985). A cellular gene encodes scrapie PrP 27-30 protein. *Cell* 40, 735-746.
- 15 Oesch, B., Jensen, M., Nilsson, P., and Fogh, J. (1994). Properties of the scrapie prion protein: quantitative analysis of protease resistance. *Biochemistry* 33, 5926-5931.
- 20 Pan, K.M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E., and Prusiner, S.B. (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci. U. S. A.* 90, 10962-10966.
- 25 Prusiner, S.B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science* 216, 136-144.
- Prusiner, S.B. (1991). Molecular biology of prion diseases. *Science* 252, 1515-1522.
- 30 Prusiner, S.B., Groth, D., Serban, A., Koehler, R., Foster, D., Torchia, M., Burton, D., Yang, S.L., and DeArmond, S.J. (1993). Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proc. Natl. Acad. Sci. U. S. A.* 90, 10608-10612.
- 35 Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R., and Wuthrich, K. (1996). NMR structure of the mouse prion protein domain PrP(121-321). *Nature* 382, 180-182.
- 40 Riesner, D., Kellings, K., Wiese, U., Wulfert, M., Mirenda, C., and Prusiner, S.B. (1993). Prions and nucleic acids: search for "residual" nucleic acids and screening for mutations in the PrP-gene. *Dev. Biol. Stand.* 80, 173-181.
- Riesner, D., Kellings, K., Post, K., Wille, H., Serban, H., Groth, D., Baldwin, M.A., and Prusiner, S.B. (1996). Disruption of prion rods generates 10-nm spherical particles having high alpha-helical content and lacking scrapie infectivity. *J. Virol.* 70, 1714-1722.
- 45 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press).

- Scharff, M.D. (1978). *Current Topics in Microbiology and Immunology* 81, 1-7.
- Serban, D., Taraboulos, A., DeArmond, S.J., and Prusiner, S.B. (1990). Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. *Neurology* 40, 110-117.
- 5 Stahl, N., Borchelt, D.R., Hsiao, K., and Prusiner, S.B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* 51, 229-240.
- 10 Stahl, N., Baldwin, M.A., Teplov, D.B., Hood, L., Gibson, B.W., Burlingame, A.L., and Prusiner, S.B. (1993). Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry* 32, 1991-2002.
- 15 Tagliavini, F., Frangione, B. (1993) Soluble prion polypeptides, and methods for detecting and purifying thereof. International patent application number: PCT//US93/04600; International publication number WO 93/23432; international publication date: 25.11.93
- 20 Takahashi, K., Shinagawa, M., Doi, S., Sasaki, S., Goto, H., and Sato, G. (1986). Purification of scrapie agent from infected animal brains and raising of antibodies to the purified fraction. *Microbiol. Immunol.* 30, 123-131.
- 25 Tobler, I., Gaus, S.E., Deboer, T., Achermann, P., Fischer, M., Rulicke, T., Moser, M., Oesch, B., McBride, P.A., and Manson, J.C. (1996). Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature* 380, 639-642.
- Wells, G.A. and Wilesmith, J.W. (1995). The neuropathology and epidemiology of bovine spongiform encephalopathy. *Brain Pathol.* 5, 91-103.
- 30 Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., and Smith, P.G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK (see comments). *Lancet* 347, 921-925.
- 35 Williams, A.E., Lawson, L.J., Perry, V.H., and Fraser, H. (1994). Characterization of the microglial response in murine scrapie. *Neuropathol. Appl. Neurobiol.* 20, 47-55.
- Williams, A.E., Ryder, S., and Blakemore, W.F. (1995). Monocyte recruitment into the scrapie-affected brain. *Acta Neuropathol.* 90, 164-169.
- 40 Williamson, R.A., Peretz, D., Smorodinsky, N., Bastidas, R., Serban, H., Mehlhorn, I., DeArmond, S.J., Prusiner, S.B., Burton, D.R., Mouse antibody libraries, Phage display, Gene ablation, Scrapie, Prion, and disease. (1996). Circumventing tolerance to generate autologous monoclonal antibodies to the prion protein. *Proceedings of the National Academy of Sciences of the United States of America* 93, 7279-7282.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Immunological Detection of Prions

(iii) NUMBER OF SEQUENCES: 9

20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
25 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: EP 97102837.8
(B) FILING DATE: 21-FEB-1997

30

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 660 base pairs
35 (B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

45

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
(D) DEVELOPMENTAL STAGE: Adult

(vii) IMMEDIATE SOURCE:

50

(B) CLONE: pbPrP3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

55

ATGAAGAAGC GACCAAAACC TGGAGGAGGA TGGAACACTG GGGGGAGCCG ATACCCAGGA 60

CAGGGCAGTC CTGGAGGCAA CCGTTATCCA CCTCAGGGAG GGGGTGGCTG GGTCAGCCC 120

CATGGAGGTG GCTGGGGCCA GCCTCATGGA GGTGGCTGGG GCCAGCCTCA TGGAGGTGGC 180
 TGGGGTCAGC CCCATGGTGG TGGCTGGGGA CAGCCACATG GTGGTGGAGG CTGGGGTCAA 240
 5 GGTGGTACCC ACGGTCAATG GAACAAACCC AGTAAGCCAA AAACCAACAT GAAGCATGTG 300
 GCAGGAGCTG CTGCAGCTGG AGCAGTGGTA GGGGGCCTTG GTGGCTACAT GCTGGGAAGT 360
 GCCATGAGCA GGCCTCTTAT ACATTTTGGC AGTGA CTATCGTGAA 420
 10 AACATGCACC GTTACCCCAA CCAAGTGTAC TACAGGCCAG TGGATCAGTA TAGTAACCAG 480
 AACAACTTTG TGCATGACTG TGTC AACATC ACAGTCAAGG AACACACAGT CACCACCACC 540
 15 ACCAAGGGGG AGAACTTCAC CGAACTGAC ATCAAGATGA TGGAGCGAGT GGTGGAGCAA 600
 ATGTGCATTA CCCAGTACCA GAGAGAATCC CAGGCTTATT ACCAACGAGG GGCAAGTTAA 660

20 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 219 amino acids
 (B) TYPE: amino acid
 25 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30 (iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 35 (A) ORGANISM: Bos taurus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

40 Met Lys Lys Arg Pro Lys Pro Gly Gly Gly Trp Asn Thr Gly Gly Ser
 1 5 10 15
 Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln
 20 25 30
 45 Gly Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro
 35 40 45
 His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro
 50 50 55 60
 His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Gly Trp Gly Gln
 65 70 75 80
 55 Gly Gly Thr His Gly Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn
 85 90 95
 Met Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly
 100 105 110

Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu Ile His
 115 120 125
 5 Phe Gly Ser Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met His Arg
 130 135 140
 Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln
 145 150 155 160
 10 Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Val Lys Glu His Thr
 165 170 175
 Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Ile Lys
 180 185 190
 Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr Gln Arg
 195 200 205
 20 Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ala Ser
 210 215

(2) INFORMATION FOR SEQ ID NO: 3:

- 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 30 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "oligonucleotide"
 (iii) HYPOTHETICAL: NO
 35 (iv) ANTI-SENSE: NO

- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGGAATTCCA TATGAAGAAG CGACCAAAAC CTG

33

45 (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid
 50 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "oligonucleotide"
 55 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
CGGGATCCTA TTAAGTTGCC CCTCGTTGGT A 31

5 (2) INFORMATION FOR SEQ ID NO: 5:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

25 Leu Ile His Phe Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

40 (iv) ANTI-SENSE: NO

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu
1 5

50 (2) INFORMATION FOR SEQ ID NO: 7:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

10 Gly Ser Asp Tyr Glu Asp Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

25 (iv) ANTI-SENSE: NO

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Tyr Tyr Arg Pro Val Asp Gln Tyr Ser
1 5

35 (2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
40 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

45 (iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

55 Cys Ile Thr Gln Tyr Gln Arg Glu Ser Gln Ala Tyr Tyr
1 5 10

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Dr. Carsten Korth
Dr. Bruno Oesch
Institut für Hirnforschung
Universität Zürich
August-Forelstr. 1
CH-8029 Zürich

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR Name: Dr. Carsten Korth Dr. Bruno Oesch Address: Institut für Hirnforschung Universität Zürich August-Forelstr. 1 CH-8029 Zürich	II. IDENTIFICATION OF THE MICROORGANISM Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2295 Date of the deposit or the transfer ¹ : 1997-02-06
III. VIABILITY STATEMENT The viability of the microorganism identified under II above was tested on 1997-02-07 ² . On that date, the said microorganism was <div style="margin-left: 40px;"> <input checked="" type="checkbox"/> viable <input type="checkbox"/> no longer viable </div>	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴ <div style="height: 40px;"></div>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): <div style="text-align: center; font-family: cursive; font-size: 1.2em;">U. Werh</div> Date: 1997-02-19

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.


⁴ Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

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Dr. Bruno Oesch
Institut für Hirnforschung
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August-Forelstr. 1
CH-8029 Zürich

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: 6H4	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2295
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I. above was accompanied by:</p> <p>() a scientific description () a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1997-02-06 (Date of the original deposit)¹.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).</p>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 1997-02-19

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.




BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Dr. Carsten Korth
Dr. Bruno Oesch
Institut für Hirnforschung
Universität Zürich
August-Forelstr. 1
CH-8029 Zürich

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR Name: Dr. Carsten Korth Dr. Bruno Oesch Address: Institut für Hirnforschung Universität Zürich August-Forelstr. 1 CH-8029 Zürich	II. IDENTIFICATION OF THE MICROORGANISM Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2296 Date of the deposit or the transfer ¹ : 1997-02-06		
III. VIABILITY STATEMENT The viability of the microorganism identified under II above was tested on 1997-02-07 ² . On that date, the said microorganism was <div style="margin-left: 40px;"> <input checked="" type="checkbox"/> viable <input type="checkbox"/> no longer viable </div>			
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴ <div style="height: 40px;"></div>			
V. INTERNATIONAL DEPOSITARY AUTHORITY <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): <div style="text-align: center; margin-top: 20px;">  </div> Date: 1997-02-19 </td> </tr> </table>		Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): <div style="text-align: center; margin-top: 20px;">  </div> Date: 1997-02-19
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): <div style="text-align: center; margin-top: 20px;">  </div> Date: 1997-02-19		

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
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Institut für Hirnforschung
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CH-8029 Zürich

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: 34C9	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2296
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I. above was accompanied by:</p> <p>() a scientific description () a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1997-02-06 (Date of the original deposit)¹.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).</p>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): <i>V. Warko</i> Date: 1997-02-19


¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Universität Zürich
Institut für Hirnforschung
August-Forelstrasse 1
CH-8029 Zürich

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Universität Zürich Institut für Hirnforschung Address: August-Forelstrasse 1 CH-8029 Zürich	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2298 Date of the deposit or the transfer ¹ : 1997-02-13
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 1997-02-14 ¹ . On that date, the said microorganism was <input checked="" type="checkbox"/> (X) viable <input type="checkbox"/> () no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 1997-02-27

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.


⁴ Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE¹

INTERNATIONAL FORM

Universität Zürich
Institut für Hirnforschung
August-Forelstrasse 1
CH-8029 Zürich

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: 15B3	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2298
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I. above was accompanied by:</p> <p>() a scientific description () a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1997-02-13 (Date of the original deposit)¹.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).</p>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 1997-02-27

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

CLAIMS:

1. A monoclonal antibody or a fragment thereof capable of specifically binding to native and denatured normal (PrP^C) and disease-specific prion protein (PrP^{Sc}) in an antigen-antibody complex.
- 5 2. A monoclonal antibody according to claim 1 which is only capable of binding to native disease-specific prion protein and not to native normal prion protein.
3. A monoclonal antibody according to claim 1 wherein the prion protein is soluble.
4. A monoclonal antibody according to claim 1 wherein the prion protein is insoluble.
- 10 5. A monoclonal antibody according to claim 1 wherein the prion protein is a recombinant prion protein.
6. A monoclonal antibody according to claim 1 wherein the prion protein is reduced.
7. A monoclonal antibody according to claim 1 wherein the prion protein is oxidized.
- 15 8. A monoclonal antibody according to claim 1 which is named 6H4.
9. A monoclonal antibody according to claim 1 which is named 34C9.
10. A monoclonal antibody according to claim 1 and 2 which is named 15B3.
11. A monoclonal antibody according to claim 1 which comprises an epitope binding fragment of anyone of the monoclonal antibodies 6H4 or 34C9 or 15B3.
- 20 12. A monoclonal according to claim 1 or 2 coupled to other molecules especially fragments of other antibodies, enzymes or organic chemical compounds.
13. An antibody raised against the binding region (idiotype) of the antibodies according to claim 1 or 2.
14. A hybridoma cell line capable of producing a monoclonal antibody according to
25 anyone of claims 1-13.
15. A hybridoma cell line according to claim 14 deposited under DSM.ACC2295 capable of producing the monoclonal antibody 6H4.
16. A hybridoma cell line according to claim 14 deposited under DSM ACC2296 capable of producing the monoclonal antibody 34C9.
- 30 17. A hybridoma cell line according to claim 14 deposited under DSM ACC2298 capable of producing the monoclonal antibody 15B3.

18. A recombinant protein derived from cloning protein-coding sequences from cell lines according to claims 15, 16, 17.

19. A recombinant protein according to claim 18 where the protein is expressed in a phage display system or any other system and affinity matured.

5 20. A recombinant expression vector for the expression of the bovine prion protein.

21. A recombinant expression vector according to claim 20 which is named pbPrP3.

22. The purified recombinant bovine prion protein in reduced or oxidized form or in form of a mixture thereof.

10 23. A recombinant protein according to claim 22, where the purified recombinant prion protein is from any species.

24. A method for the production of an antibody according to claim 1, comprising culturing a hybridoma cell line according to claim 14 and isolating the monoclonal antibody from the supernatant.

15 25. A method for the production of a hybridoma cell line according to claim 14, comprising administering to PrP^{0/0} mice (knockout mice without a functional PrP gene) an immunizing amount of a prion protein according to claim 22 or 23, removing the spleen from the immunized mice, recovering splenocytes therefrom, fusing the latter with a myeloma cell line, growing the fused cells in a selection medium, screening the antibodies in the supernatants of hybridoma cells for binding to native disease-specific and recombinant
20 Pr P and isolating the hybridoma cells producing monoclonal antibodies according to claim 1.

26. A method for the production of antibodies according to claims 1 or 2 comprising administering an immunizing amount of a prion protein according to claim 22 or 23 to PrP^{0/0} mice.

25 27. A method for the production of a hybridoma cell line according to claim 26 where the immunized species is a wild-type mouse, a transgenic mouse or any other wild-type or transgenic species.

28. A method for the production of an expression vector according to claim 20, comprising inserting a DNA coding for the bovine PrP in the correct reading frame into an
30 expression vector.

29. A method for the production of a purified bovine PrP protein comprising culturing a microorganism or eukaryotic cell line with an expression vector according to claim 20 in an appropriate culture medium and isolating and purifying the protein.

30.. A test kit for the diagnosis of prion diseases comprising one or more
5 monoclonal antibodies according to claim 1, purified recombinant bovine PrP protein according to claim 23, nitrocellulose sheets, microtiter plates coated or covalently linked with monoclonal antibodies according to claim 1, an antibody that is coupled with an enzyme and its substrate for a detection reaction, proteinase K, blocking buffer, homogenisation buffer and a detailed description of how to perform the test.

10 31. A test kit according to claim 30 comprising a nitrocellulose membrane in the dipstick format coated with an antibody according to claim 1 or 2, a dilution buffer, a solution containing an antibody according to claim 1 or 2, coupled to colloids evoking a colouring reaction when present in an antigen-antibody complex, and a detailed description of how to perform the test

15 32. An immunological detection procedure for the detection of disease-specific PrP in biological material of an animal or human comprising treatment of a probe of said material with proteinase K and then with the monoclonal antibody according to claim 1 or 2, detecting the prion protein-antibody complex and analysing the results.

20 33. An immunological detection procedure according to claim 32 comprising treatment of a probe of said material with the monoclonal antibody according to claim 2 without prior treatment with proteinase K, detecting the prion protein-antibody complex and analysing the results.

34. A method according to claims 30 to 32 where instead of using a monoclonal antibody recombinant prion protein according to claims 22 and 23.

25 35. A pharmaceutical preparation for the therapy and prevention of prion diseases comprising a monoclonal antibody or fragments thereof according to claim 1 or 2 and a pharmaceutical carrier.

30 36. A method for the therapy or prevention of prion diseases comprising administering to a patient suffering from such disease or being likely to becoming a victim of this disease a therapeutical or preventive amount of a monoclonal antibody according to claim 1 or 2.

37. A method for clearing biological material from prions comprising treating said material with a monoclonal antibody according to claim 1 or 2.

38. A method for the therapy or prevention of prion diseases, or the vaccination against prion diseases comprising administering to a patient or an animal suffering from such a diseases or being likely to becoming a victim of this disease a therapeutical or preventive amount of recombinant PrP or fragments thereof according to claim 22 or 23.

- 5 39. Use of a monoclonal antibody according to claims 1 or 2 or recombinant PrP or fragments thereof according to claim 22 or 23 for the production of a medicine or a vaccine for the therapy or prevention of prion diseases, or the vaccination against prion diseases.

Figure 1

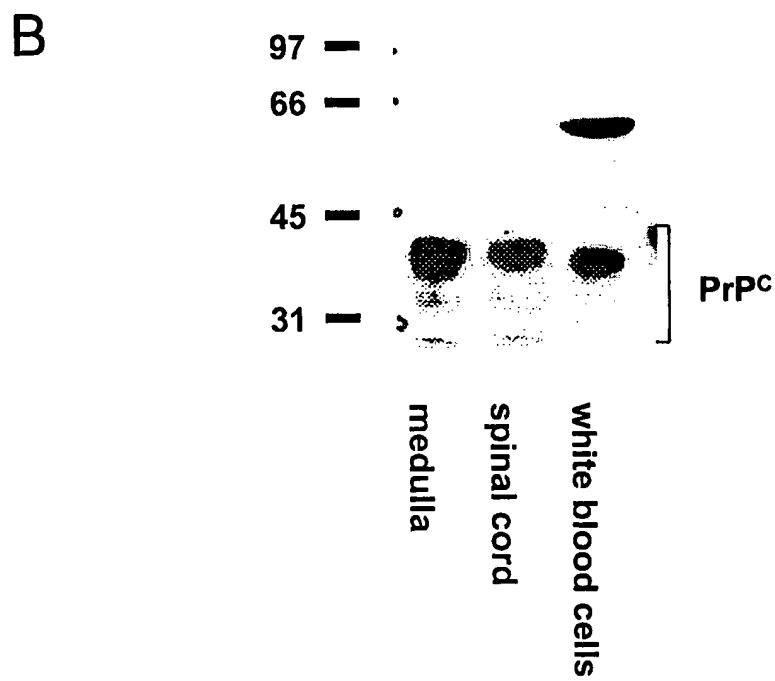
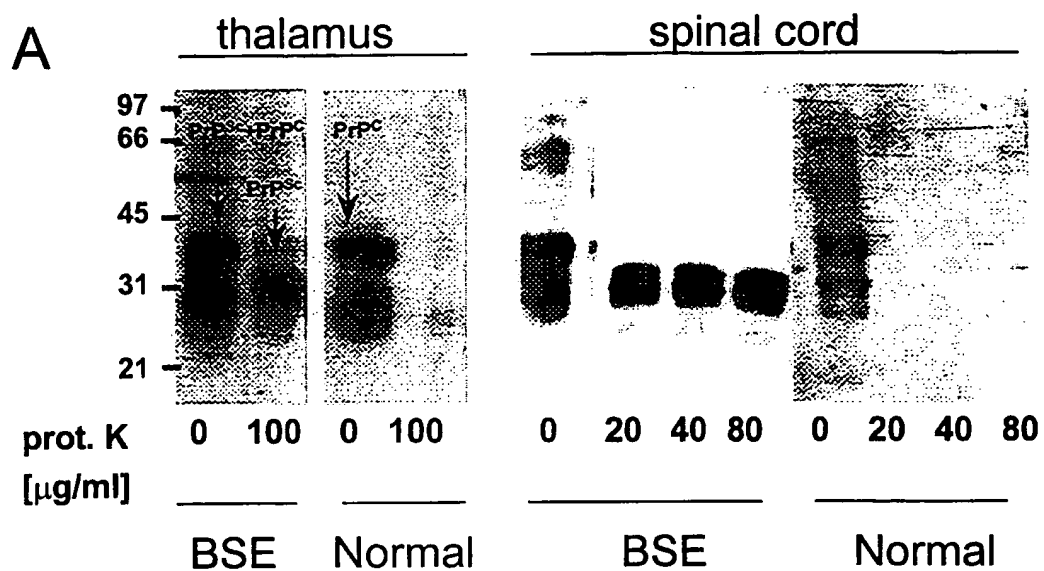


Figure 2

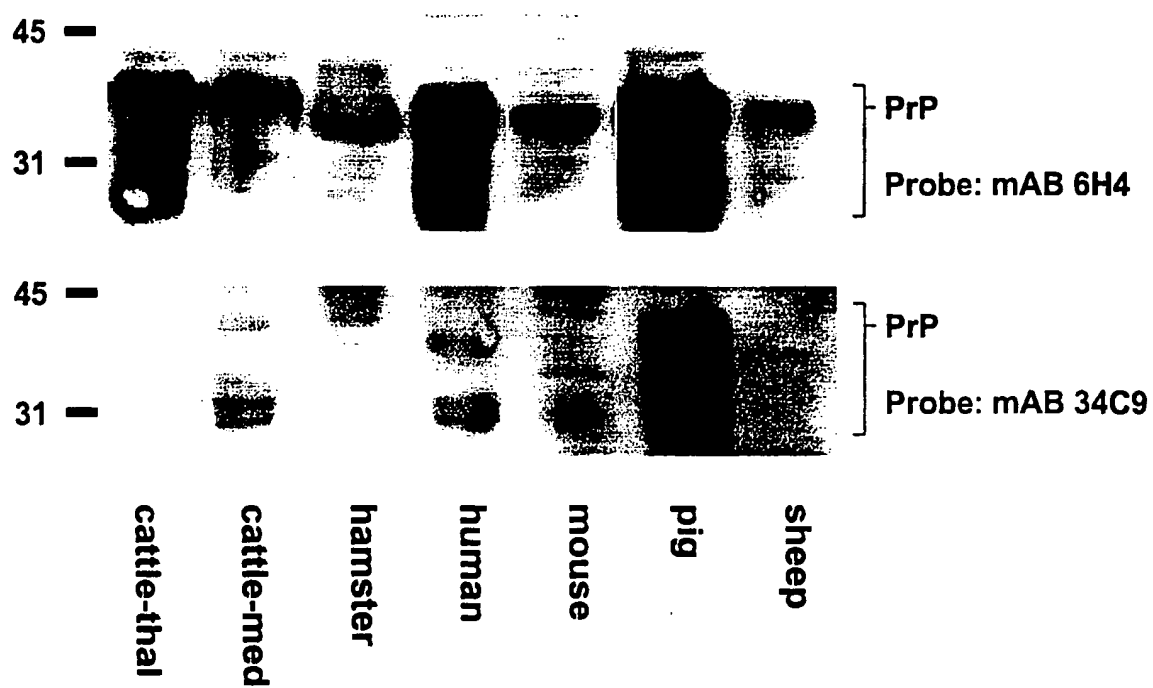
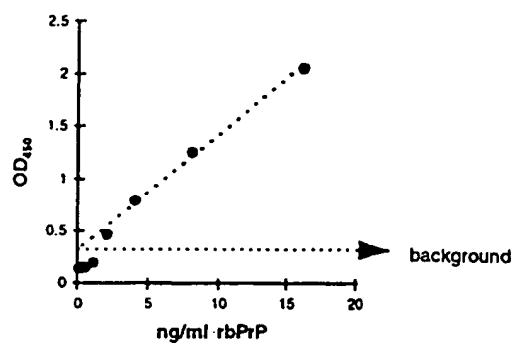


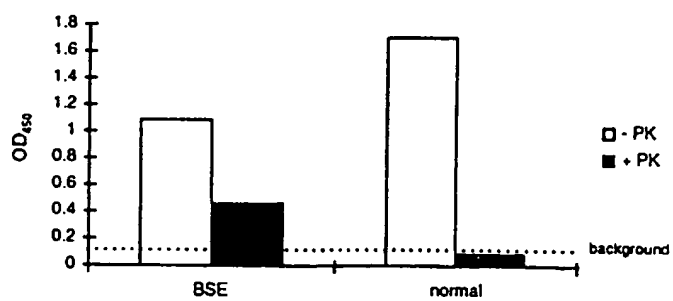
Figure 3

Standard-curve of ELIFA

a.

ELIFA for the detection of bovine PrP^{Sc}

b.

ELISA for the detection of bovine PrP^{Sc}

c.

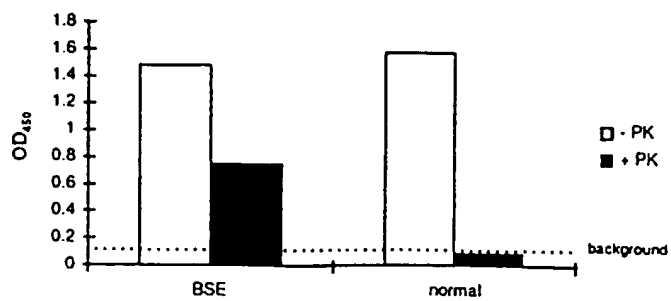


Figure 4

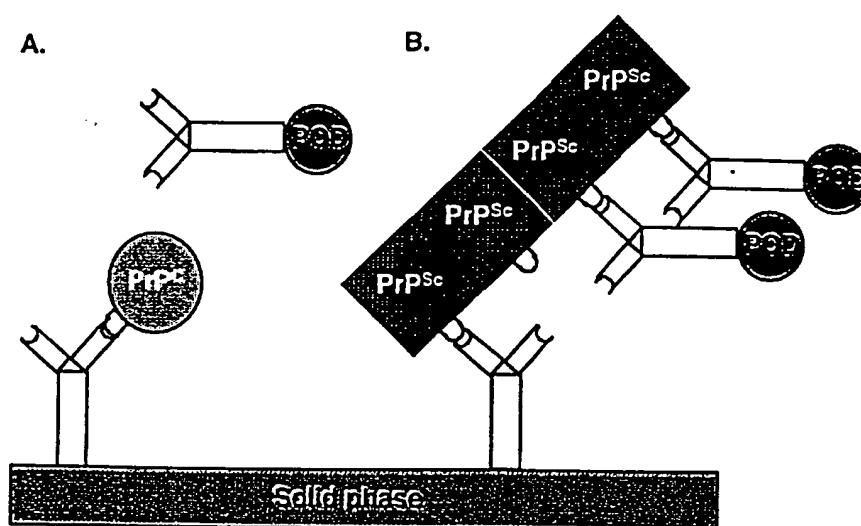
Capture-ELISA and multimeric PrP^{Sc}

Figure 5

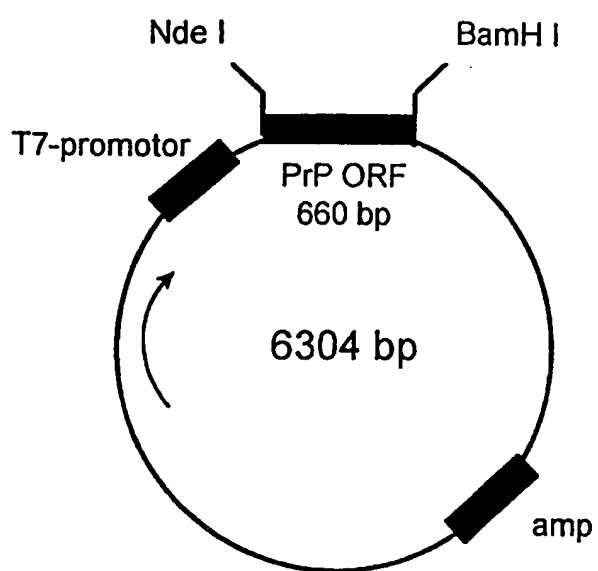
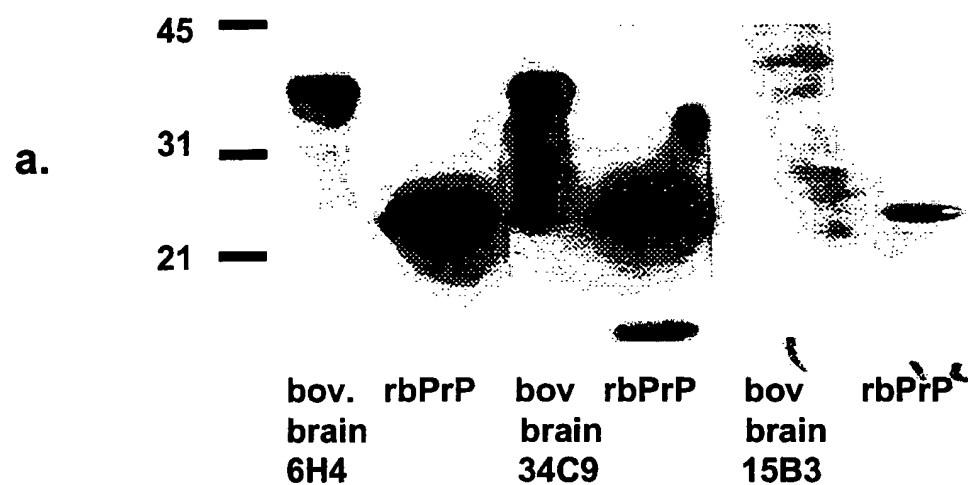


Figure 6



b.

BSE normal

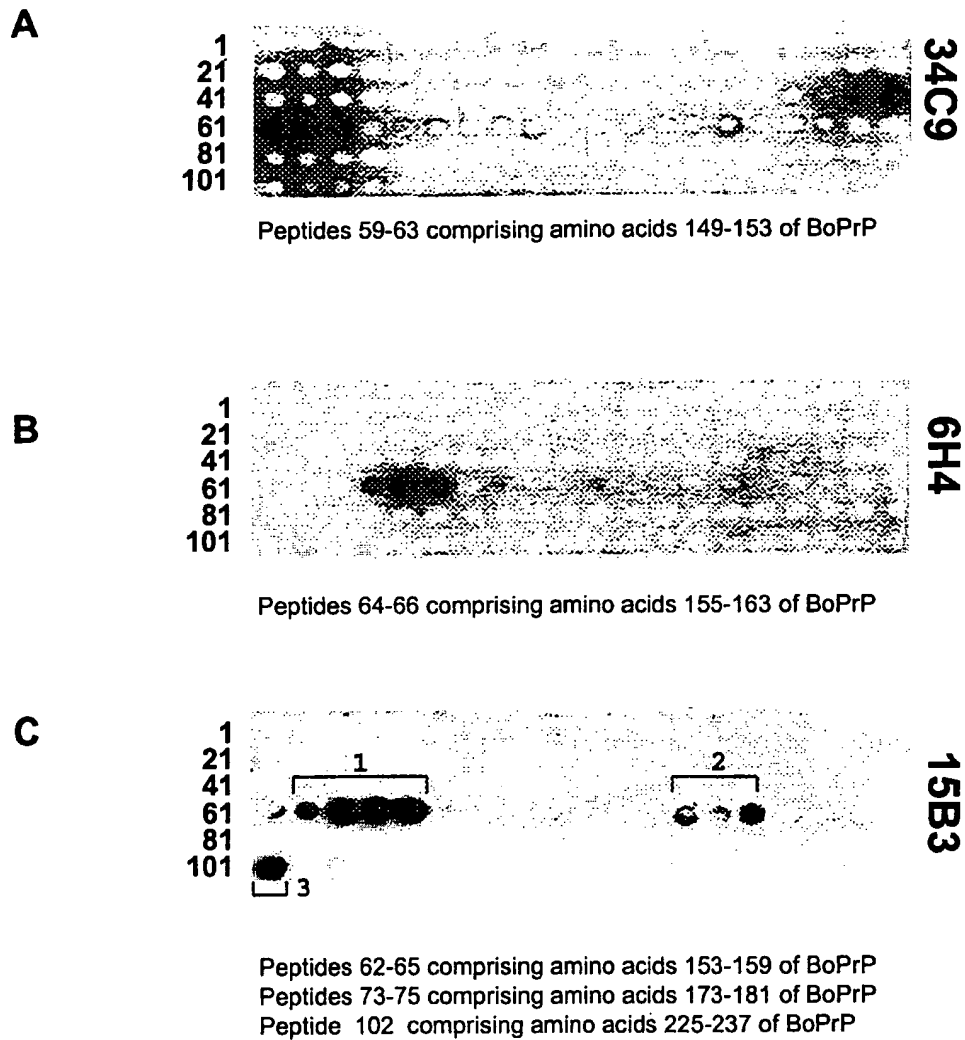
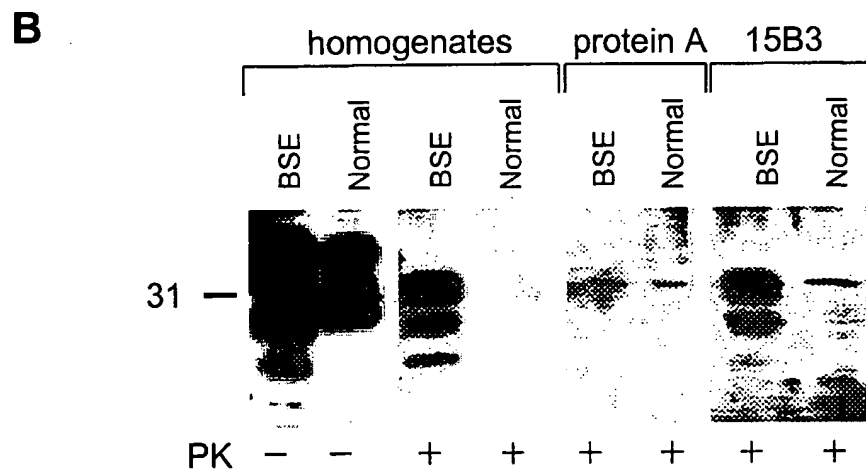
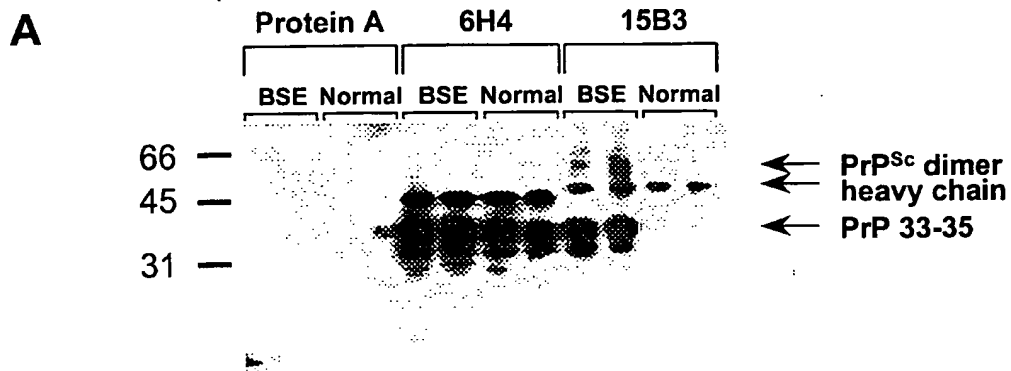
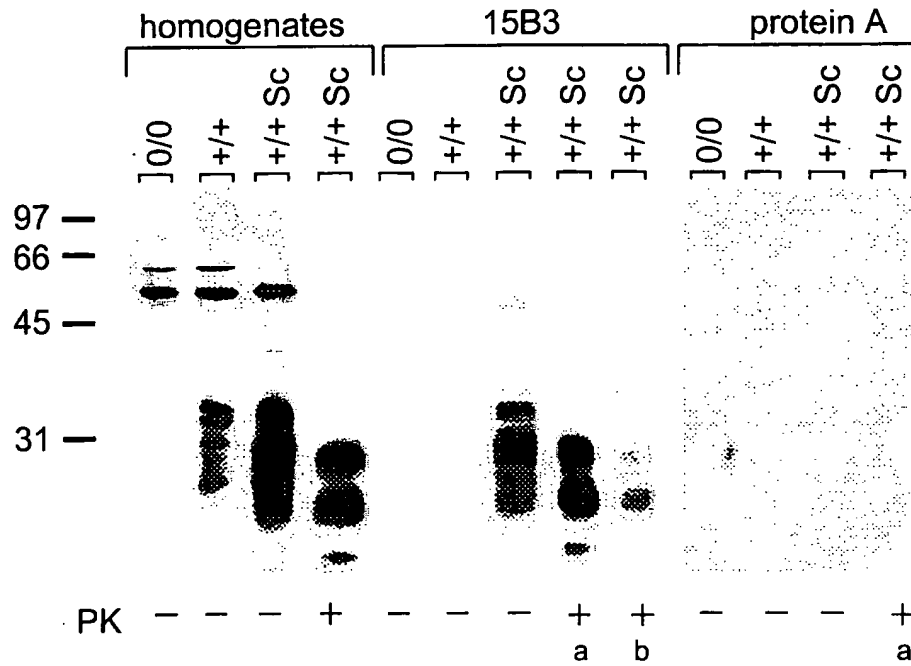
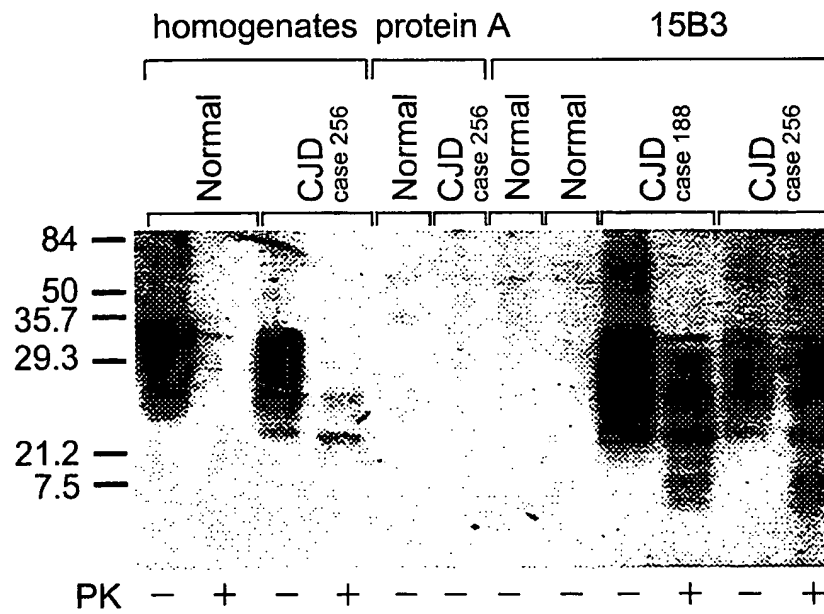
Figure 7

Figure 8

9/9

C**D**

INTERNATIONAL SEARCH REPORT

II International Application No
PCT/EP 98/00917

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/70 C07K16/18 C12N5/20 C07K14/47 C12N15/06
C12N1/21 C12N5/10 G01N33/577 G01N33/68 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,0	C. KORTH ET AL.: "Characterization of native and recombinant prion protein isoforms by differential exposure of antibody epitopes." SOCIETY FOR NEUROSCIENCE, ABSTRACTS, vol. 22, no. 1-3, 16 - 21 November 1996, page 2159 XP002036864 see abstract 844.3 ---	1-7, 32-34
X	R. KASCSAK ET AL.: "The role of antibodies to PrP in the diagnosis of transmissible spongiform encephalopathies." DEVELOPMENTS IN BIOLOGICAL STANDARDIZATION, vol. 80, 1993, BASEL, pages 141-151, XP002036865 see the whole document ---	1-7, 32-34
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

26 June 1998

Date of mailing of the international search report

15. 07. 1998

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Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/00917

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 23432 A (NEW YORK UNIVERSITY ET AL.) 25 November 1993 see the whole document ---	1,3, 32-34
A	A. PARAF: "Scrapie and bovine spongiform encephalopathy: immunological properties and diagnosis for food products." LAIT, vol. 76, no. 6, November 1996, PARIS, pages 571-578, XP002036866 see the whole document ---	1-39
A	S. PRUSINER ET AL.: "Immunologic and molecular biologic studies of prion proteins in bovine spongiform encephalopathy." JOURNAL OF INFECTIOUS DISEASES, vol. 167, March 1993, pages 602-613, XP002036867 see the whole document ---	1-39
A	M. CANN ET AL.: "Antibody fragments to PrP generated using phage display technology." JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT, vol. 18 part D, 1994, page 198 XP002036868 see abstract T304 ---	1-39
A	B. SCHREUDER ET AL.: "Preclinical test for prion diseases." NATURE, vol. 381, no. 6583, 13 June 1996, LONDON, page 563 XP000579899 see the whole document ---	1-39
A	S. KRASEMANN ET AL.: "Induction of antibodies against human prion proteins (PrP) by DNA-mediated immunization of PrP ⁰ /0 mice." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 199, no. 2, 15 December 1996, AMSTERDAM, pages 109-118, XP002036869 see the whole document ---	1-39
A	WO 93 11155 A (PROTEUS MOLECULAR DESIGN LTD.) 10 June 1993 see the whole document ---	1-39
-/--		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/00917

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	C. KORTH ET AL.: "Prion (PrPSc)-specific epitope defined by a monoclonal antibody." NATURE, vol. 390, no. 6655, 6 November 1997, LONDON, GB, pages 74-77, XP002069611 see the whole document -----	1-8, 10-12, 14,15, 17-39
P,X	WO 97 10505 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 20 March 1997 see examples see claims -----	1-4, 24-27, 29,32,33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/00917

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 36 and 38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/00917

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9323432 A	25-11-1993	AU 4376093 A	13-12-1993
WO 9311155 A	10-06-1993	AU 675053 B	23-01-1997
		AU 3089292 A	28-06-1993
		CA 2124953 A	10-06-1993
		EP 0616613 A	28-09-1994
		JP 7501798 T	23-02-1995
		NZ 246059 A	28-08-1995
		ZA 9209392 A	27-07-1993
WO 9710505 A	20-03-1997	AU 7073596 A	01-04-1997

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